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Desulfurization of Dibenzothiophene by *Pseudomonas fluorescens* (UCP 1514) Leading to the Production of Biphenyl

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Abstract

Dibenzothiophene (DBT) is a typical recalcitrant thiophenic sulfur component of fuels, and its desulphurization has been a model reaction in the treatment of these compounds. Based on this information, the potential of *Pseudomonas fluorescens* (UCP 1514) on the desulfurization of dibenzothiophene was studied, in order to use it for reducing the sulfur content of diesel oil in compliance with environmental regulations. The result of biodegradation by the bacteria was determined by undertaking high-performance liquid chromatography of the metabolites produced. These can also be identified by gas chromatography with a mass spectrometry detector, and doing so revealed a sulfur-free product, biphenyl, as the final product of the degradation process. The results showed a decrease of 73% in dibenzothiophene content, which means that *P. fluorescens* removes sulfur from dibenzothiophene with a good selectivity to form biphenyl. These promising results indicate that *P. fluorescens* has an interesting potential to degrade sulfur-containing compounds in diesel oil and thereby could help in removing sulfur content from diesel oil. The process of microbial desulfurization described herein can be used particularly after carrying out hydrodesulfurization. Consequently, the sulfur content could be reduced even further. Applying *P. fluorescens* UCP 1514 in dibenzothiophene could help to understand the nature of the biodegradation process and to achieve the regulatory standards for sulfur level in fossil fuels.

Keywords: biodegradation, biodesulfurization, dibenzothiophene, diesel, *Pseudomonas fluorescens*

1. Introduction

Direct combustion of fossil fuels leads to sulfur oxide emissions that contribute to acid rain and air pollution. Dibenzothiophene (DBT) and its derivatives are the main organic sulfur compounds found in petroleum. These compounds are present due to the pathway by which petroleum is formed [1].

Various types of heterocycles containing oxygen, nitrogen, and sulfur are found in the environment, originating from anthropogenic or natural sources. Dibenzofurans, dibenzodioxins, and dibenzothiophene are among the most important environmental pollutants and are well covered in the literature [2].

As these heterocyclic compounds are relatively stable, the traditional processes, such as acid alkali treatment, are inefficient at removing them from petroleum.

Hydrogenation has been widely used for petroleum desulfurization. Because of the rigorous operating conditions (high pressure and temperature in the presence of a catalyst) and the consumption of hydrogen, this technique can be expensive both in the capital investment needed and in its operating costs [3].

Several microorganisms are used to remove sulfur compounds which are recalcitrant to HDS, such as dibenzothiophene (DBT) and its alkylated derivatives [4]. Degradation aided by microbes is a viable bioremediation technology for organic pollutants. There is a wide range of microorganisms which could be involved in the breaking of chemical bonds. So, bioremediation uses the metabolic versatility of microorganisms to degrade hazardous pollutants [5].

Biodesulfurization (BDS) is one of the most promising technologies that is used together with the traditional hydrodesulfurization (HDS) to reduce the sulfur content in fossil fuels [6]. The limited efficiency of the HDS method leads to residues which may contain thiophenic compounds. Most of the sulfur remaining in a fuel after a HDS process is in the form of thiophenic compounds, of which dibenzothiophene (DBT) is a typical recalcitrant. Therefore desulfurizing DBT has been used as a model reaction when treating fossil fuels [7].

Research on biodesulfurization using DBT has resulted in two different biochemical pathways, named Kodama [8] after their author and 4S [9]. The Kodama pathway is considered unsuitable for treating fuel because water-soluble sulfur compounds are produced, which are thus unavailable for burning and so reduce the calorific value of the fuel.

On using the 4S pathway, DBT is transformed into 2-hydroxybiphenyl (2-HBP) and sulfite. In this pathway, the carbon skeleton of DBT is released intact under the formation of 2-HBP; therefore, its calorific value is not lost [10].

We report experiments of bioremediation of DBT by *Pseudomonas fluorescens*. This microorganism was found to degrade DBT via the sulfur-selective pathway and hence was applied so as to desulfurize diesel oil. The bacteria succeeded in appreciably decreasing the sulfur in DBT, thus indicating their potential for use as biocatalysts in desulfurizing fossil fuels.

2. Materials and methods

2.1. Microorganism and culture conditions

The experiments were carried out using a pure culture of bacterium *P. fluorescens* (UCP 1514) belonging to the Nucleus for Research in Environmental Sciences (NPCIAMB), of the Catholic University of Pernambuco. The bacterial culture was maintained in solid medium nutrient agar consisting of meat extract (5.0 g); peptone (10.0 g); NaCl (5.0 g); agar (15.0 g/l of distilled water), to which was added by syringe; and 2 mM DBT dissolved in dimethylformamide (DMF), at 4°C as a stock of bacterial inoculum.

2.2. Fermentation conditions

The culture was grown in Luria-Bertani (LB) medium containing tryptone (10.0 g), yeast extract (5.0 g), NaCl (10.0 g), and glucose (5.0 g/l) as supplement, as per Konishi et al. [11]. The following DBT concentration and solvent was used as stock solution: 1 M in dimethylformamide (DMF) [12]. The solution was sterilized in a Millipore® filter.

The Erlenmeyer flasks were autoclaved at 120°C for 30 min. Batch kinetic experiments were carried out in 250 mL Erlenmeyer flasks by adding 2 mM DBT solution to 100 mL LB liquid culture medium.

Another culture was prepared as inoculum just before batch experiment, and while the bacteria were in the exponential phase, a volume of 5 mL consisting of 108 cells/mL was transferred to 100 mL of liquid medium. All experiments were made in triplicate followed by control trials without inoculum, performed in a rotary shaker operating at 150 rpm at a constant temperature of 37°C for 144 h. Aliquots were obtained every 24 h.

Bacterial growth was monitored through biomass and pH using a pH meter (Orion 310). The specific growth rate (μ_{esp}) and generation time (T_G) were determined according to [13]. For the specific growth rate, Eq. (1) was used:

$$\mu_{esp} = \frac{\ln X - \ln X_0}{T - T_0}$$

Where,

X = Final biomass
 X_0 = Initial biomass
 T = Final time
 T_0 = Initial time

(1)

The generation time was determined by:

$$T_G = \ln_2 / \mu_{esp}$$

After cultivation, the samples were centrifuged at 5000 × g for 15 min at 5°C to separate the biomass and metabolic liquid. After separation the biomass was lyophilized to determine the dry weight of biomass per unit volume.

2.3. HPLC and GC-MS analysis

In order to determine the intermediates of the pathway of DBT biodesulfurization by microorganisms, the bacterial culture was grown at 37°C in a rotary shaker at 150 rpm for different periods and was then centrifuged at $5000 \times g$ for 15 min at 5°C to separate the biomass and metabolic liquid.

The supernatant was extracted with an equal volume of ethyl acetate. The organic layer was removed, and the aqueous layer was acidified with 5 N HCl to pH 2.0 and again extracted with an equal volume of ethyl acetate.

The two extracts were pooled, evaporated to dryness under vacuum in a rotavapor system and resuspended in 1 mL of ethyl acetate. DBT was quantified by HPLC analysis, for which the organism was grown in LB containing 2 mM DBT for different time periods, mixed with an equal volume of ethyl acetate and analyzed by using an HPLC Varian UV-VIS detector, model 320. Separation was carried out with a C18 RP column (4.6×250 mm), solvent liberation system, model 210 Varian Star®, with the following separation conditions: mobile phase acetonitrile 75% and phosphate buffer 10 mM (pH 6.0). The eluate was detected at 232 nm [14].

Gas chromatography (GC) analysis was performed in a Varian Star 3600CX with a DB-1 (100% dimethylpolysiloxane) fused capillary column ($30 \text{ m} \times 0.25 \text{ mm}$); column temperatures were programmed from 50°C for 5 min, raised to 10°C/min, and then increased to 250°C for 5 min, with a total time of 30 min for integrating purposes.

Injector and detector temperatures were 250°C. 3.0 μL of a solution of about 2 mM DBT in ethyl acetate was injected. DBT analysis was carried out using a Varian Star CX 3600 equipped with Varian Saturn 2000. The carrier gas was helium 5.0 WHITE MARTINS, MS split dibenzothiophene WAX. Mass spectra were taken at 70 eV. Scanning speed was 1.5 scans/s from m/z 40 to 500.

The samples containing DBT were also analyzed by GC-MS; identification was made by the computerized matching of the mass spectra obtained with those stored in the MAINLIB library of the GC-MS data system and by the Spectral Database for Organic Compounds SDBS library.

3. Results

3.1. Effects of DBT on the growth of *P. fluorescens*

P. fluorescens UCP 1514 grew in the control as well as in DBT medium (**Figure 1**). The beginning of the exponential phase was observed between 24 and 72 h of cultivation. The growth rate of the control was 0.65 h^{-1} , and in experiments with DBT, it was 0.2 h^{-1} , with a generation time of 0.027 and 0.006 h in the control and in the DBT culture, respectively (**Table 1**).

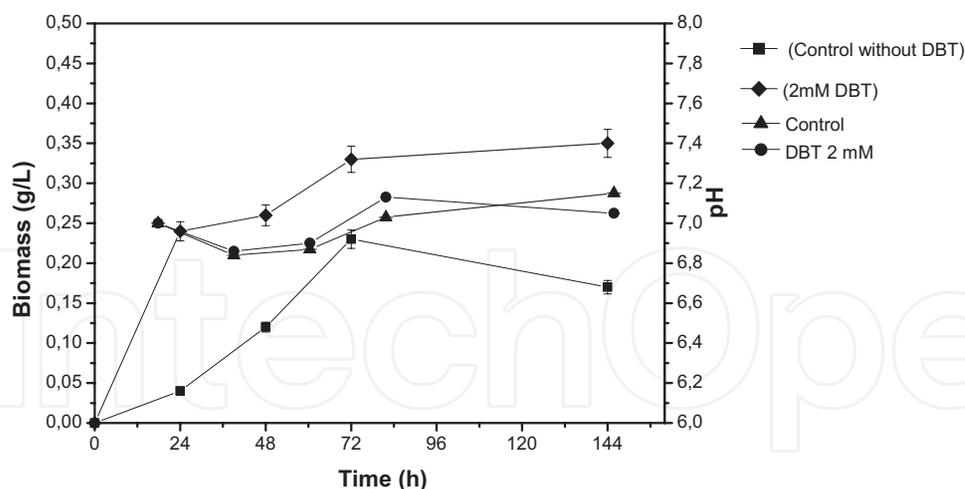


Figure 1. Growth and pH of *Pseudomonas fluorescens* cultured in Luria-Bertani medium with 2 mM dibenzothiophene (DBT) as sole sulfur source. (●) pH with DBT, (■) pH control, (▲) biomass with DBT, and (■) biomass control.

The pH did not decrease during cultivation. With the control, growth was observed until 72 h of cultivation with 0.23 g dry cell/L. After this period, the decline phase of microorganisms began, with 0.17 g dry cell/L at 144 h. However, in experiments with DBT, the opposite occurred; after adaptation time, the population of the microorganism continued to increase, presenting 0.35 g dry cell/L at 144 h as well as maintaining it, probably due to using hydrocarbon and its metabolites as the main energy source [15].

3.2. The use of DBT by *P. fluorescens*

To study the ability of the bacteria to use DBT in liquid cultures, the amount of DBT in the cultures was measured by HPLC as a function of time. DBT utilization and its decrease over time by *P. fluorescens* are shown in **Figure 2**. Biodegradation started after 48 h of culture, presenting a decrease on the initial DBT concentration from 2 to 1.98 mM and at the end of fermentation fell to 0.54 mM, having metabolized about 73% of DBT.

Desulfurizing DBT by *P. fluorescens* and determining pathway intermediates

In order to determine the pathway intermediates of DBT degradation by *P. fluorescens*, GC-MS of ethyl acetate extracts of cultures grown over different periods were performed and revealed several peaks (**Figures 3–5**). Besides the solvent peaks, three peaks were further analyzed by their mass spectra to deduce the structures.

Experiments	μ_{esp}	T_G
Control	0.65 h ⁻¹	0.027 h
Medium with 2 mM DBT	0.32 h ⁻¹	0.006 h

Table 1. Values of μ_{esp} and T_G during kinetics growth of *P. fluorescens*.

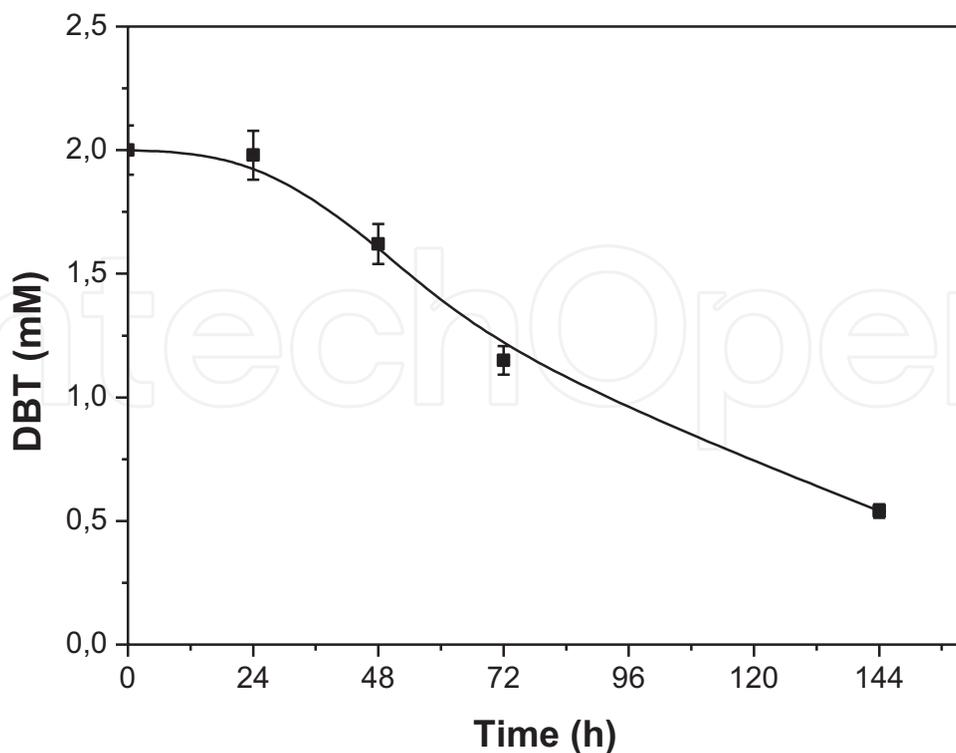


Figure 2. Time course of dibenzothiophene (DBT) utilization by *Pseudomonas fluorescens* (UCP 1514A) cultured in Luria-Bertani medium with 2 mM DBT as sole sulfur source.

As shown in **Figures 3–5**, three compounds were detected as metabolites of DBT (16.7 min, $m/z = 184$); one metabolite was identified as benzoic acid (11.9 min, $m/z = 105$), the second as methanecarbothiolic acid (0.66 min, $m/z = 42$), and the third as biphenyl (12.3 min, $m/z = 154$). Based on the structures of the metabolites (**Figure 6**) and the DBT-desulfurizing pathway [16], we suggest that DBT (1) degradation by *P. fluorescens* should begin by identifying dibenzothiophene sulfone with degradation to benzoic acid and changes from methanecarbothiolic acid to 2-mercaptobenzoic acid (2).

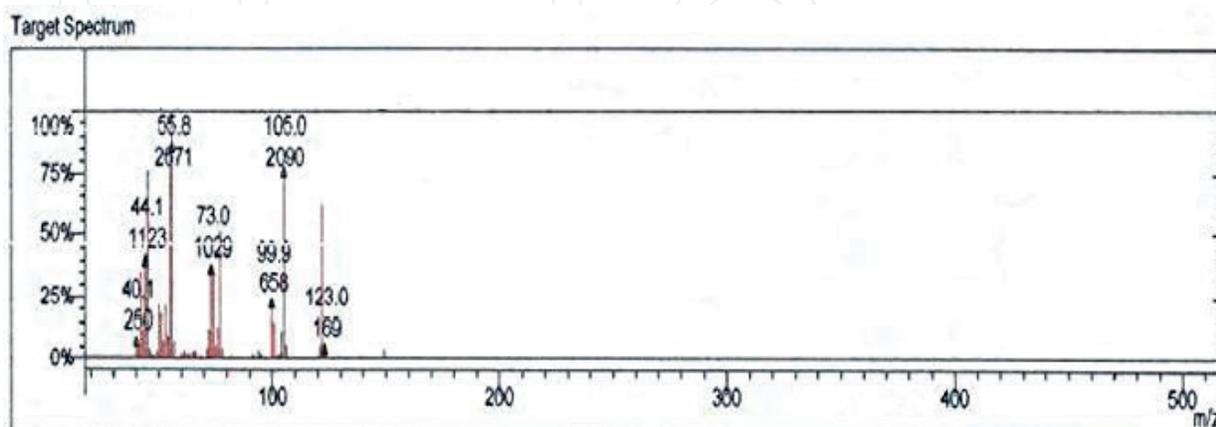


Figure 3. Representative mass spectra of dibenzothiophene in *Pseudomonas fluorescens*. (A) Benzoic acid, (A.1) benzoic acid (Spectral Database for Organic Compounds SDBS library).

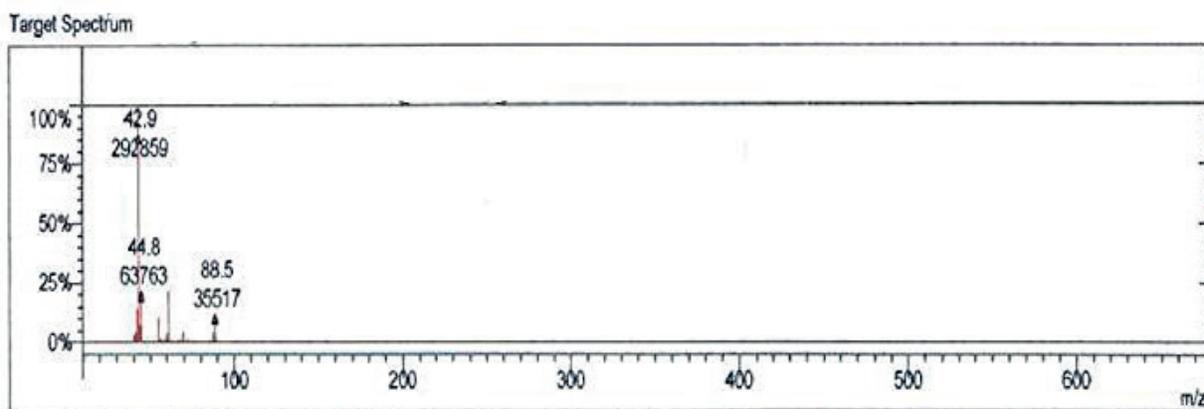


Figure 4. Representative mass spectra of dibenzothiophene in *Pseudomonas fluorescens*. (B) Methanecarbothiolic acid, (B.1) methanecarbothiolic acid (Spectral Database for Organic Compounds SDBS library).

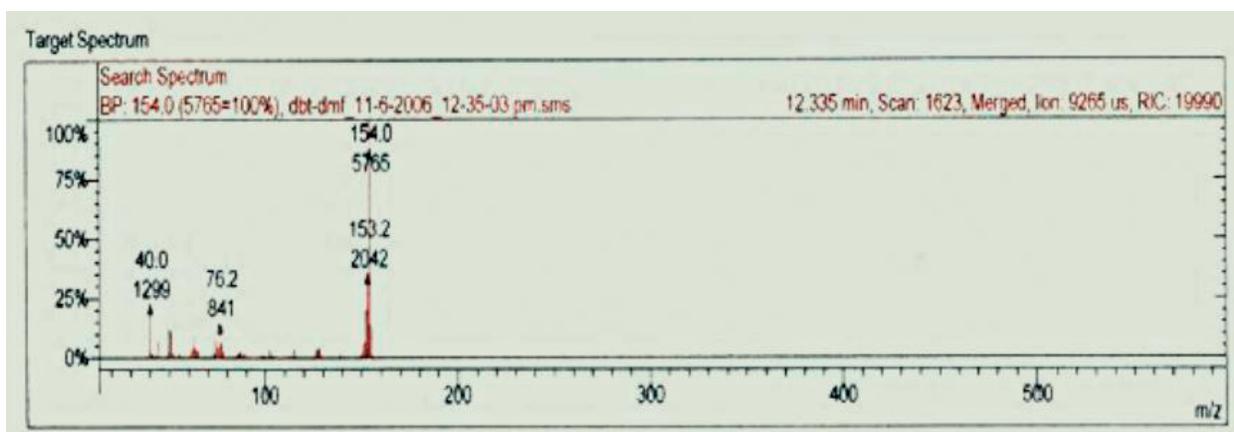


Figure 5. Representative mass spectra of dibenzothiophene in *Pseudomonas fluorescens*. (C) Biphenyl, (C.1) biphenyl (Spectral Database for Organic Compounds SDBS library).

According to [17], metabolite (3) had a molecular ion at m/z 216 and fragment ions at m/z 200 and 184 produced by two sequential losses of O ($M+ -16$ and -32) and thus was identified as dibenzothiophene sulfone (Table 2, Figure 6).

The detection of dibenzothiophene sulfone demonstrates the presence of the step of sulfur oxidation. According to [11], metabolite (4) was assumed to be dibenzo[*c*] [1,2]oxathiin S-oxide ($m/z = 166$). The abundance of fragment ions at ($m/z = 182$) may be due to loss of S = O from the molecular ion. Metabolite (5) was deemed to be dibenzo[*c*] [1,2]oxathiin S,S-dioxide. DBT was transformed to DBT sulfone [18], and a ring cleavage product 4-methoxybenzoic acid (6) was detected.

This fragmentation could be explained by the loss of oxygen after a loss of carbonyl. According to [1] metabolite (7) was assumed to be 2-HBP ($m/z = 170$); metabolite (8) was assigned as 2-methoxybiphenyl (2-MBP) ($m/z = 184$), and the last metabolite was designated as biphenyl ($m/z = 154$) (9), corresponding to loss of hydrogen and phenol group formation.

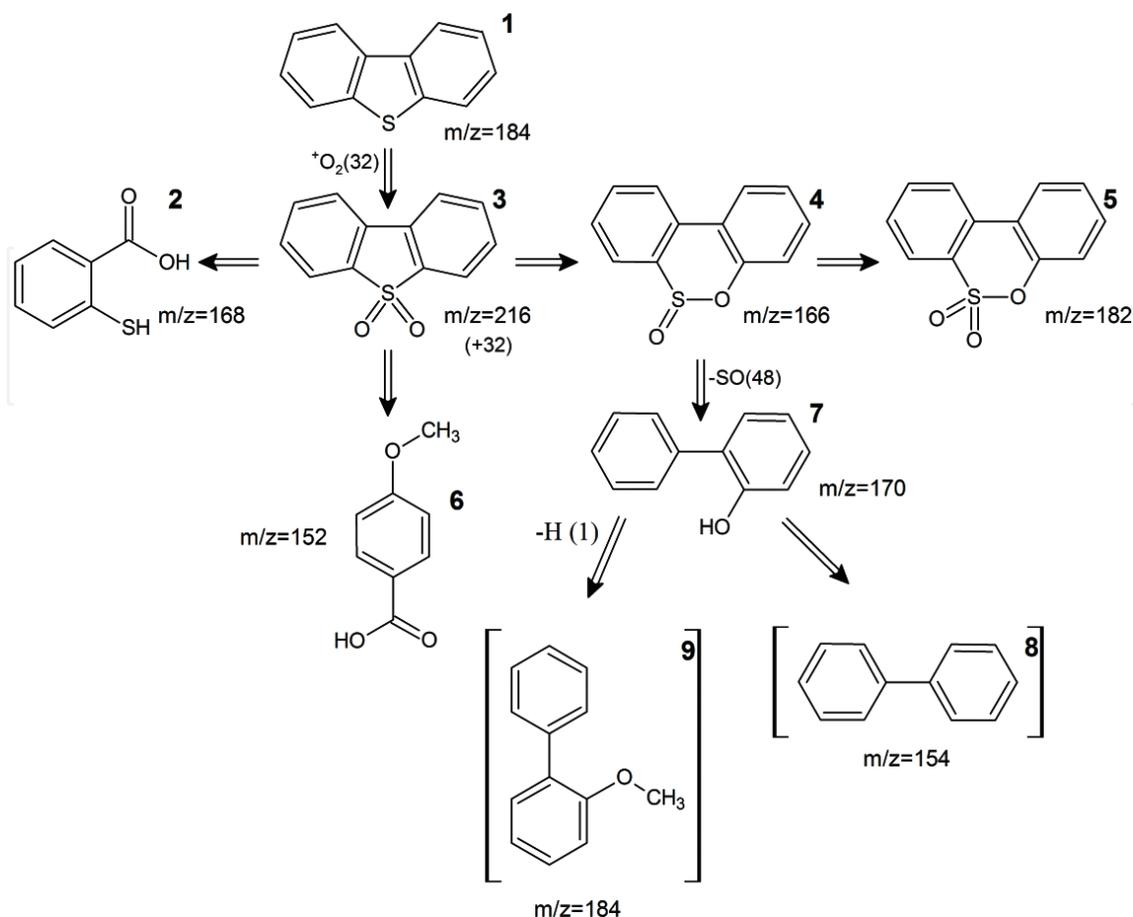


Figure 6. Proposed catabolic pathways of dibenzothiophene by *Pseudomonas fluorescens* (UCP 1514A). (1) Dibenzothiophene, (2) 2-mercaptobenzoic acid (Me), (3) dibenzothiophene 5,5'-dioxide, (4) dibenzo[e][1,2]oxathiin S-oxide, (5) dibenzo[c][1,2]oxathiin S,S-dioxide, (6) 4-methoxybenzoic acid, (7) 2-hydroxybiphenyl, (8) biphenyl, and (9) 2-methoxybiphenyl. The structures in brackets were the metabolites detected; the other structures are proposed intermediates or metabolites, but not detected.

ID	Fragment ion m/z (relative abundance, %)	Compound	Molecular ion m/z
1	185 (14.6), 184 (100), 139 (10.8), 92 (7.8)	Dibenzothiophene	184
2	165 (36), 136 (100), 108 (35)	2-Mercaptobenzoic acid (Me)	168
3	216 (14), 200 (17), 184 (100)	Dibenzothiophene sulfone	216
4	118 (100), 90 (47), 63 (33), 39 (20), 48 (19), 166 (9), 77 (5), 110 (4), 138 (3)	Dibenzo[c][1,2]oxathiin S-oxide	166
5	89 (100), 118 (99), 182 (73), 63 (65), 43 (38), 134 (25), 51 (22), 71 (18), 97 (7), 109 (5), 150 (4)	Dibenzo[c][1,2]oxathiin S,S-dioxide	182
6	152 (74.6), 135 (100), 107 (14.5), 92 (10), 77 (20.5)	4-Methoxybenzoic acid	152
7	171 (12.8), 170 (100), 141 (23.8), 169 (46.5), 115 (16.7)	2-Hydroxybiphenyl	170
8	186 (1.2), 185 (14.2), 184 (100), 169 (42.8), 141 (25.7), 139 (8.6)	2-Methoxybiphenyl	184
9	154 (100), 152 (18), 151 (5.1), 76.2 (10.3)	Biphenyl	154

Table 2. Mass spectral characteristics of proposed degradation products.

4. Discussion

P. fluorescens UCP 1514 remained viable in the presence of DBT, probably due to this element being considered essential for the formation of amino acids such as cysteine, and methionine, some vitamins, and other compounds that are important for the survival of microorganisms [19].

The development of BDS has been prompted by increasingly stringent regulations regarding limiting the sulfur content in transportation fuels. BDS is based on 4S pathway. When the pathway is used, DBT is not degraded, but it is transformed into 2-HBP which partitions in the oil phase, resulting in no loss of caloric value of the fuel [10]. DBT-containing hydrocarbon was desulfurized by growing cells of *Pseudomonas* sp. [20], as well as by growing and resting cells of *Gordonia* sp. at 30°C [21]. Martinez et al. [22] studied the biodesulfurization of dibenzothiophene by resting cells of *Pseudomonas putida* CECT5279, where complete conversion into HBP was reached in 90 min, using a gas flow of 2 vvm, thus showing that the 4S route is highly sensitive to oxygen availability working under oxygen-limiting conditions.

Several authors stated that the initial degradations of anthracene and other compounds do not depend on lignolytic activity and suggested that cytochrome P-450 monooxygenase could be responsible for this initial step [23].

The catabolic pathway of DBT was proposed, based on the metabolites tentatively identified by their mass spectra (**Figure 6**). The analysis of DBT metabolites suggested that *P. fluorescens* UCP 1514 can decompose DBT by multiple pathways. Ref. [24] reported dibenzothiophene 5-5'-dioxide, which is involved in the sulfur-specific process of biodesulfurization named as 4S pathway [7]. In this study, DBT degradation by *P. fluorescens* started from dibenzothiophene sulfone which was probably further metabolized to 2-mercaptobenzoic acid, according to [24]. Ref. [25] detected several DBT metabolites including benzo[b]thiophene-2,3-diol, 2-mercaptobenzoic acid, and 2,2'-dithiosalicylic acid from *P. fluorescens* 17 and 26 cultures.

The dibenzothiophene was transformed to DBT sulfone and from this compound to dibenzo[c][1,2]oxathiin S-oxide and dibenzo[c][1,2]oxathiin S,S-dioxide or metabolized to 4-methoxybenzoic acid, a ring cleavage product [26]. The conversion of DBT S,S-dioxide into dibenzo[c][1,2]oxathiin S-oxide could be explained by the oxidative cleavage of one of the two C-S bonds in DBT S,S-dioxide and circularization of the cleavage product under acidic conditions. However, it is likely that DBT sulfone is also an intermediate compound in the bacterial DBT degradation pathway. No peak corresponding to DBT sulfone was detected in our analysis. Benzo[c][1,2]oxathiin S-oxide was also found as the metabolite from benzothiophene in *Gordonia* sp. 213E strain [27]. This can be formed from (Z)-2-(2'-hydroxyphenyl)ethene-1-sulfinate, the thiophene ring-open form, under acidic extraction conditions [11]. As shown in **Figure 6**, two possible metabolites, not including any sulfur in their molecular structures, were presented at the end of the biodegradation pathway of DBT.

Based on the deduced structures such as 2-MBP and biphenyl, it is interesting to note that *P. fluorescens* (UCP 1514) can produce two extra sulfur-free metabolites. In addition, according to the molecular structure of the two metabolites, it is presumed that they might be further produced from 2-HBP or from the intermediate metabolite HPBS in parallel with 2-HBP.

It was also supposed that 2-MBP was formed by the methylation of the hydroxyl group of 2-HBP [28]. These results suggest that producing 2-MBP and biphenyl has the advantage of partially eliminating the enzyme inhibitory effect of 2-HBP, thereby prolonging desulfurizing activities. Therefore, further studies investigating the enzymatic and genetic properties of UCP 1514 are being carried out to explore the possibilities of inducing this strain to form more 2-MBP and biphenyl. As reported, 2-HBP was toxic to bacterial cells, and once the concentration of 2-HBP was above 0.2 mM, the biodesulfurization of DBT was inhibited [29].

The inhibitory effects of 2-HBP, the end product of the desulfurization of dibenzothiophene (DBT) by the 4S pathway, were noted early in the study of biodesulfurization [30–32]. However, product inhibition has not been fully appreciated as the main impediment to developing improved biocatalysts, and effective means of overcoming the inhibition caused by 2-HBP are just beginning to be developed [33]. Only about half of the DBT consumed can be accounted for as 2-HBP in some biodesulfurization studies, implying that 2-HBP accumulates intracellularly [34–37]. This results in some increase in the activity of the 4S pathway. Moreover, the high enzymatic activities reported for the compartmentalization of the reaction steps of the 4S pathway illustrate that the inhibition by 2-HBP of desulfurization enzymes and the metabolism of cells strongly influence the desulfurization activity of biocatalysts [33]. Because 2-HBP strongly inhibits the 4S pathway, there is advantage in selecting any culture that alleviates/overcomes this inhibition. Among the numerous biodesulfurization cultures isolated, there are several that possess an extended 4S pathway such that 2-HBP is not the end product of DBT desulfurization, and instead 2-methoxybiphenyl (2-MBP) [38–40], biphenyl [41], or 2,2-dihydroxybiphenyl [42] are produced. The toxicity of 2-MBP is reported to be only slightly less than 2-HBP [43], but this study examined the inhibition of DBT biodesulfurization by externally added 2-MBP or 2-HBP and did not address the intracellular accumulation of 2-HBP [44].

In biodesulfurization, DBT is converted to 2-HBP, which increases the possibility of environmental pollution [45]. In contrast, the production of 2-MBP and biphenyl may partially eliminate the inhibitory effect of products and pollution from diesel oil combustion. In this study, intermediate metabolites such as DBTO, DBTO₂, and HPBS were not detected by GC-MS analysis, which could be explained by the lability of desulfurized DBT metabolites [16] or the existence of an additional degradation pathway for DBT [46].

The capabilities of the isolated bacteria to survive and desulfurize a wide range of S compounds present in crude oil are desirable traits for the development of a robust BDS biocatalyst to upgrade crude oils and refinery streams [47]. Therefore, UCP 1514 desulfurizes DBT using a sulfur-specific degradation pathway, with selective cleavage of the C-S bonds, and is considered to be novel and different from other pathways.

Further work is required to find out the actual mechanism of DBT metabolism. There are some technologies that oil refineries use to remove sulfur, such as oxidative desulfurization (ODS), photocatalytic desulfurization, and HDS. HDS involves catalytic treatment of fuel at high temperatures (>300°C) and pressures (>100 atm) to remove the bulk of inorganic sulfur. Simple organic sulfur can be removed by HDS, but this process is inadequate for producing low-sulfur fuels as it is unable to remove the complex polycyclic sulfur compounds present in petroleum and coal.

Oxidative desulphurization (ODS) enables ultra-low-sulfur content to be attained in diesel fuels by oxidizing refractory sulfur compounds that are difficult to remove with hydrodesulphurization when the sulfur content to be attained needs to be below 10 mg/kg [48]. On using ODS, sulfur-containing hydrocarbons can be only oxidized to sulfoxides and sulfones using H₂O₂ as oxidants in acetonitrile as solvents [49].

Desulfurization can be performed efficiently by irradiation combined with a chemical catalyst. But the consumption of chemicals seemed to be excessive, which would result in a higher cost, according to [3].

Until now, conventional refining processes have been performed at much higher temperatures. Therefore thermophilic biodesulfurization is desirable without cooling the stock to 30°C [50]. Moreover, thermophilic biodesulfurization also reduces the viscosity of crude oil, which makes the process more practicable [51].

5. Conclusion

P. fluorescens UCP 1514 desulfurizes DBT through a sulfur-specific degradation pathway, with selective cleavage of the C-S bonds, and is considered to be novel and different from other pathways. Further work is required to find out the actual mechanism of DBT metabolism. The results indicate that *P. fluorescens* could have a good potential to be used in biocatalytic desulfurization of fossil fuels. Further work aimed at developing the strain is underway.

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