Chapter 15

Reduction in the Sulfur Content of Fossil Fuels by *Cunninghamella elegans* (UCP 0596) to Dibenzothiophene Compound

Patrícia Mendes de Souza,
Thayse Alves de Lima e Silva,
Marcos Antonio Barbosa Lima,
Luciana de Oliveira Franco, Manfred Schwartz,
Paulo Henrique da Silva, Lúcia Roberta Barbosa,
Aline Elesbão do Nascimento, Kaoru Okada and
Galba Maria de Campos-Takaki

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http://dx.doi.org/10.5772/intechopen.70432

Abstract

Biodesulfurization (BDS) is one of the most promising technologies used together with traditional hydrodesulfurization (HDS) to reduce the sulfur content of fossil fuels. In this research study, a strain of *Cunninghamella elegans* (UCP 596) was isolated from mangrove sediments to metabolize an organosulfur dibenzothiophene (DBT) compound in the concentrations of 0.5 and 1 mM and transform to DBT sulfone (DBT-5-dioxide), followed by dibenzothiophene 5,5-dioxide and 2-hydroxybiphenyl metabolites, thus suggesting the use of the "4S" metabolic pathway. The fungus also degraded the DBT completely in the first 24 h of growth on a 2.0 mM DBT concentration by angular deoxygenation, which suggests that a new second metabolic pathway was used. The DBT was consumed as the carbon source, and the sulfur was removed in the form of sulfite ion. A new product, benzoic acid, was formed at the end of the catabolism of DBT by *C. elegans* using an angular route.

Keywords: *Cunninghamella elegans*, biodesulfurization, dibenzothiophene, angular deoxygenation

1. Introduction

Environmental pollution, acid rain, and health problems are caused when sulfur dioxide is emitted into the atmosphere as the result of the combustion of petroleum fractions. To solve these problems, regulations are increasingly stringent in order to minimize the levels of sulfur emitted into the atmosphere. Hydrodesulfurization (HDS) is a conventional technology used to remove sulfur from fossil fuels. This is achieved by using metal catalysts and hydrogen gas, but despite the extremely high pressures and temperatures, the process does not eliminate heterocyclic organosulfur compounds, especially those such as dibenzothiophene (DBT) [1–3]. Hydrodesulfurization is a very effective technique for removing thiols, sulfides, and disulfides, but it is not suitable for removing thiophene compounds. Therefore, petrochemical industries have sought techniques that enable the sulfur to be removed from these heterocyclic compounds [4].

An alternative is biodesulfurization (BDS), a more efficient and low-cost process, which uses microorganisms to desulfurise these compounds, by promoting selective metabolism of the sulfur (attacking C-S) without degrading the carbon skeleton (CC), thus keeping the energy source of the molecule intact. Dibenzothiophene is considered a model compound for studying the biological desulfurization of fossil fuels and persistent compounds such as S-heterocycles in the environment [4, 5].

Several microorganisms have been studied with a view to using them to remove sulfur biochemically from DBT. Prokaryotic organisms that desulfurise organosulfur compounds without metabolizing the carbon skeleton are uncommon and are usually used in ways that seek to oxidize sulfur selectively [5–12].

Eukaryotic organisms, such as *Cunninghamella elegans*, grow on DBT by forming DBT-5-oxide and DBT-5-dioxide, but biphenyl is not formed [13]. *Trichosporon* sp. uses phenol and dibenzothiophene as the only source of carbon and sulfur, respectively, and studies on it have shown that it has the capacity to transform biphenyl and dibenzothiophene, and therefore could be used to remove these toxic compounds [14].

Trametes trogii UAMH 8156, *Trametes hirsuta* UAMH 8165, *Phanerochaete chrysosporium* ATCC 24725, *Trametes versicolor* IFO 30340 (formerly, *Coriolus* sp.), and *Tyromyces palustris* IFO 30339 all oxidized DBS to dibenzyl sulfoxide prior to oxidation to dibenzyl sulfone [15].

The fungus *Paecylomyces* sp. specifically removes sulfur by oxidizing DBT and produces 2,2′-dihidroxibifenil [16, 17]. Biological oxidation of sulfur is primarily catalyzed by two enzymatic systems, P450 monooxygenases and flavin-containing mono oxygenases (FMO). However, in most organisms, the enzymes responsible for oxygenating sulfur (S-oxygenation) have not been clearly identified. *C. elegans* catalyzes the S-oxygenation of DBT; consequently, oxygenases were identified as being responsible for the S-oxygenation of DBT in *C. elegans* [18].

Dibenzothiophene is a heterocycle compound that is regarded as the most potent environmental pollutant. Microbial degradation of this pollutant is attractive, and the bioprocesses for DBT biodegradation are environment friendly. This study focuses on investigating the biotechnological potential of *C. elegans* UCP 0596 to degrade DBT and the products formed.

http://dx.doi.org/10.5772/intechopen.70432

2. Materials and methods

2.1. Preserving the microorganism

A microorganism was isolated from mangrove sediment of the Rio Formoso, Pernambuco, Brazil. The fungus was identified as *C. elegans* (UCP-596), deposited in the Culture Collection of the Catholic University of Pernambuco, and registered in the World Federation for Culture Collection-WFCC. The fungus was maintained on potato dextrose agar (PDA) medium at 5°C and transferred to a new medium every 4 months.

2.2. Chemicals

The DBT was purchased from Aldrich, cat: D3, 220-2 and a stock solution prepared in NN-dimethylformamide at a concentration of 1 M (w/v). The solution was sterilized in a Millipore® filter, as described by Araújo et al. [22]. All other chemicals were of analytical grade. All organic solvents were of HPLC grade (E Merck).

2.3. Inoculum and culture conditions

Inoculum from these actively growing cultures was used. *C. elegans* was grown in Potato Dextrose Agar medium (PDA) at 28°C during 6 days, until sporulation, and after this period, spore suspension was prepared containing 107 sporangioles/mL. For the assay to biodegrade dibenzothiophene in *C. elegans*, 500 mL Erlenmeyer flasks containing 200 mL of Sabouraud liquid medium (control) were used. The medium was treated with dibenzothiophene in concentrations of 0.50, 1.0, and 2 mM and inoculated with 5% of sporangiole suspension, after which the mixture was incubated in an orbital shaker at 150 rpm, at 28°C, and aliquots were collected every 24 h until 96 h of growth. Thereafter, a metabolic liquid was obtained by centrifugation at 5000 g for 15 min at 5°C to separate the biomass.

2.4. Extraction of the metabolite produced

The metabolic liquid was extracted by chromatography, using the method described by Labana et al. [19]. To determine the intermediate compounds in the metabolic pathway used by *C. elegans* to degrade DBT, the supernatants were extracted with an equal volume of ethyl acetate (60 :60 mL). The organic layer was removed, and the aqueous layer was acidified to pH 2.0 with 5 N HCl solution, and extracted with an equal volume of ethyl acetate. The extracts were dried using a rotor, evaporated in a vacuum, and re-suspended with 2 mL of ethyl acetate and analyzed by gas chromatography-mass spectrometry (GC-MS).

2.5. Analysis using gas chromatography-mass spectrometry (GC-MS)

The analysis was conducted by using a Varian Star 3600 CX Gas Chromatograph, coupled to a Varian Saturation 2000 Mass Spectrometer, with a CP-WAX 58 FFAP-CB column, 50 m, 0.32 mm ID, DF = 0.2 mm. The carrier gas used was White Martins helium 5.0, Pressure 8 PSI. The programmed temperature was 50°C for 5 min, which was increased by 10°C min^{-1},

until the temperature reached 250°C for 5 min, with a total time of 30 min. The temperature of the injector and detector was 250°C. Approximately 3.0 μL of each solution extracted with ethyl acetate was injected into the chromatograph. Spectrometry was performed at 70 eV. The scan speed was 1.5 scans s^{-1} at 40–500 m/z. The samples containing DBT were also analyzed using the GC/MS. DBT was identified by comparing the mass spectro obtained in the MAINLIB library of the GC/MS system and the Spectral Database for Organic Compounds SDBS library.

2.6. Determining the removal of DBT

The level of removal of DBT from a solution of 1–10 mM DBT was determined by using a UV Sensor to visualize a curve with at a wavelength of 250 nm running through the metabolic liquid for different concentrations of DBT (0.5, 1.0, and 2.0 mM) at a constant temperature of 28°C and shaken at 150 rpm for 144 h. Measurements were made every 24 h [20].

3. Results and discussion

3.1. Biodegradation of DBT by *C. elegans*

The remarkable ability of fungi to survive in different niches is a consequence of the evolution of enzyme systems, which have coexisted for billions of years with an enormous variety of natural substances of different origins. This diversity of substrates, which have the potential for microbial growth in hydrophobic sources, induced the production of enzymes that are suitable for transforming organic molecules with very different structures. Enzyme "arsenals" have even been able to act on synthetic chemical substances that are derived from human activities. When there are hydrophobic sources, there is no doubting that the response of the metabolism of certain microorganisms gives some additional advantages to microbial cells. This includes exploiting new ecological niches as energy sources [14]. Among fungi, *C. elegans* has been reported as having the ability to oxidize and degrade several Polycyclic Aromatic Hydrocarbons (PAHs) such as anthracene, acenaphthene, benzo(a)anthracene, benzo(a)pyrene, phenanthrene, fluoranthene, and naphthalene, as well as nitrated hydrocarbons, which are considered to be mutagenic and carcinogenic agents [15].

In this study, a strain of *C. elegans* (UCP 0596) grown on Sabouraud culture medium supplemented with 0.50 mM DBT was able to degrade about 70% of DBT in 24 h of growth. In the end, about 97% of DBT of the growing cell of *C. elegans* was removed after 96 h of cultivation, and the residual DBT was determined as being 0.015 mM (**Figure 1**). The results showed that a DBT degradation of 0.50 and 1.0 mM occurred due to the function of the concentrations and the elapsed time. On the other hand, in *C. elegans* grown on Sabouraud medium containing only 1.0 mM DBT as source, the concentration decreased to 44% in the 24 h of growth. At the end of cultivation, the content of DBT was reduced to 0.019 mM, which corresponds to biodegradation of 98.1%. The life of the DBT 2 mM by *C. elegans* records a decrease in the amount of DBT, 100% of which has been removed at 24 h, for the concentrations studied (**Figure 1**).

http://dx.doi.org/10.5772/intechopen.70432

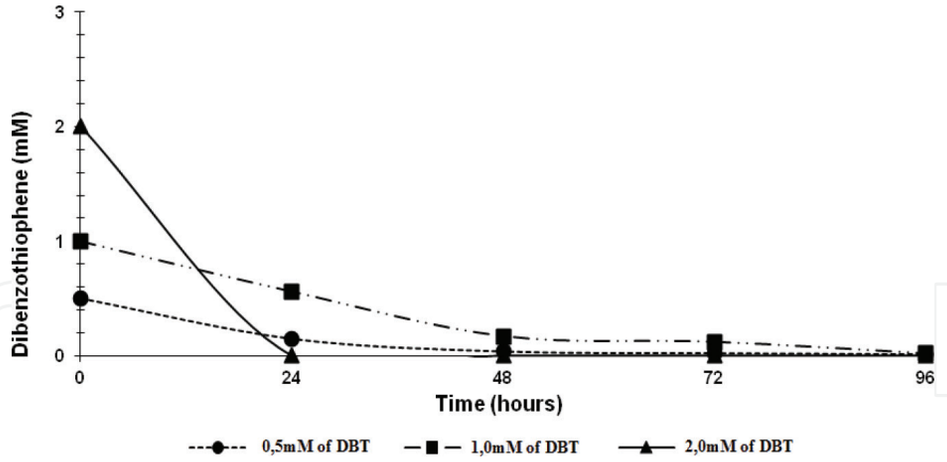

Figure 1. Degradation of dibenzothiophene by *Cunninghamella elegans* (UCP 596) over time in liquid Sabouraud culture medium, containing 0.50, 1.0, and 2.0 mM of DBT, at 28°C, for 96 h, at 150 rpm.

However, the environmental problems with DBT degradation have received much more attention from researchers worldwide. Organic compounds containing sulfur are a small but important fraction of some fuels and due to it being difficult to biodegrade them, they are considered to be recalcitrant compounds. The presence of sulfur is undesirable because it contributes to the corrosion of equipment in the refinery, and also to the emission of sulfur oxides (SOx) into the atmosphere by the combustion of oil, thus causing environmental problems, such as air pollution and being a potential cause of acid rain [17].

The metabolites produced by biodegradation of *C. elegans* were identified as dibenzothiophene 5-oxide, dibenzothiophene 5,5-dioxide in the first 48 h of growth and 2-hydroxybiphenyl within 72 h of growth. These results suggest the fungus used the "4S" metabolic pathway. The results are in agreement with studies by Schlenk et al. [13].

However, although these studies detected several products because DBT was biodegraded, desulfurization with the formation of 2-hydroxybiphenyl was not observed. In our results, dibenzothiophene dioxide 5-5, a compound found in the "4S" pathway, was found.

This pathway is one strategy for reducing these emissions, namely to remove sulfur from mineral carbon, petroleum and its derivatives before combustion. Currently, physical and chemical processes deemed to be hydrodesulfurization (HDS) ones are being used in refineries to remove inorganic sulfur (**Table 1**). These treatments incur very high costs since they involve using chemical catalysts under extreme conditions (200–425°C) and high pressures of from 150 to 205 psi [1]. Inorganic sulfur and organic sulfur can be removed by HDS, but this process is unsuitable for producing fuels with low sulfur content since this process is unable to remove sulfur compounds from complex polycyclical hydrocarbons, containing sulfur, which are present in petroleum and coal. Thus, tiophenic compounds represent a large amount of sulfur after treatment of HDS in fuels. Another strategy to reduce the sulfur content is to expose these subtracts to microorganisms that can specifically break the carbon-sulfur chain, thus releasing sulfur to a water-soluble portion, in an inorganic form. This process of microbial desulfurization or biodesulfurization (BDS) is an effective and low-cost technique [1, 2, 21].

Hydrodesulfurization (HDS)	Most commonly used method in the petroleum industry to reduce the sulfur content of crude oil. In most cases, HDS is performed by co-feeding oil and H_2 to a fixed-bed reactor packed with an appropriate HDS catalyst.
Extractive desulfurization	It is a liquid-liquid extraction process and the two liquid phases must be immiscible. It depends on the solubility of the organosulfur compounds in certain solvents.
Ionic liquid extraction	It is an interesting alternative to provide ultra clean diesel oils.
Adsorptive desulfurization	It depends on the ability of a solid sorbent to selectively adsorb organosulfur compounds from the oil.
Oxidative desulfurization (ODS)	It involves a chemical reaction between an oxidant and sulfur that facilitates desulfurization. ODS is a field of considerable interest at present.
Autoxidation	Refers to oxidation by atmospheric oxygen, i.e., oxygen in air.
Chemical oxidation	The use of a peroxide species avoids the initiation period associated with the slow in situ formation of hydroperoxides by autoxidation. The sulfur-containing compounds can directly be oxidized by the hydroperoxide to yield a sulfoxide and then a sulfone.
Catalytic oxidation	Reduce the energy barrier of oxidation by facilitating the oxidation reaction itself on the catalytically active surface; some materials serve as oxygen carriers and are more active oxidation agents than oxygen; some catalysts facilitate the decomposition of hydroperoxides, thereby accelerating the propagation step in the oxidation reaction.
Ultrasound oxidation	Provides energy for the oxidation process by ultrasound, but it does not affect the oxidation chemistry.
Photochemical oxidation	It has a high efficiency and requires mild reaction conditions. The method involves two steps: first, sulfur compounds are transferred from the oil into a polar solvent and then the transfer is followed by photooxidation or photodecomposition under UV irradiation.
Biodesulfurization (BDS)	Biodesulfurization takes place at low temperatures and pressure in the presence of microorganisms that are capable of metabolizing sulfur compounds. It is possible to desulfurize crude oil directly by selecting appropriate microbial species
Aerobic biodesulfurization	Aerobic BDS was proposed as an alternative to hydrodesulfurization of crude oil.
Anaerobic biodesulfurization	The main advantage of anaerobic desulfurization processes over aerobic desulfurization is that oxidation of hydrocarbons to undesired compounds, such as colored and gumforming products, is negligible.
Alkylation-based desulfurization	It has been tested with thiophenic sulfur compounds at small scale, and it is commercially applied for light oil at large scale as the olefinic alkylation of thiophenic sulfur (OATS) process developed by British Petroleum.
Chlorinolysis-based desulfurization	Chlorinolysis involves the scission of C–S and S–S bonds through the action of chlorine.
Supercritical water-based desulfurization	The effect of supercritical water (SCW) on desulfurization of oil is marginal. The purpose of using SCW (critical point of water: 374°C and 22.1 MPa) as reaction medium is to break C–S bonds.

Source: Javadli and Klerk [42].

Table 1. Technologies used for sulfur reduction in oil and gas industry.

3.2. Pathways for biodegradation of DBT by *C. elegans*

On the basis of these findings, the highest DBT biodegradation was observed in **Figure 2** which shows that the fungus *C. elegans* used the angular deoxygenating pathway. The fungus used DBT as a carbon source, removes the sulfur, and formed sulfite and sulfate, and at the end of biodegradation benzoic acid was produced. In this study, the DBT was catabolized by *C. elegans* for dibenzothiophene 5-oxide and dibenzothiophene 5,5-dioxide compounds in the first 48 h of growth, culminating in the formation of benzoic acid, within 72 h of growth.

The use of gas chromatography coupled to mass spectrometry identified the possible products obtained from metabolizing DBT by *C. elegans*. The catabolic pathway of DBT was proposed based on the metabolites shown in **Table 2** and the mass spectrograms in **Figure 3**. Analysis of the metabolites showed that *C. elegans* (UCP 596) can degrade DBT using two metabolic pathways.

Figure 3 confirmed the four compounds found were: dibenzothiophene 5-oxide (38.7 min, m/z = 200.2); dibenzothiophene 5,5-dioxide (29.1 min. m/z = 216.1) 2-hydroxybiphenyl (22.4 min., m/z = 170.2), and benzoic acid (22.3 min., m/z = 105.1), which were detected in the degradation of DBT (26.7 min, m/z = 184), by *C. elegans* (UCP 596).

The literature describes three well-known pathways in the process of degrading DBT by microorganisms: Kodama; angular dioxygenation; and the sulfoxide, sulfone, sulfonate, sulfate pathway deemed the "4S" [6, 9, 18–20, 23]. **Figure 4** shows the proposed routes of microbial degradation.

Dahlberg et al. [24] reported that DBT 5,5-dioxide is involved in the process of biodesulfurization by the 4S pathway. Degradation pathways of DBT by bacteria have been widely studied as in: Brevibacterium [7], Arthrobacter [24, 25], Mycobacterium [26–29]. *Pseudomonas delafieldii* and *P. putida* mineralized DBT completely, by using it as a source of carbon, sulfur, and energy, and formed as final compounds: benzoic acid, sulfite, and water by the pathway known as angular dioxygenation [30–32]. Under aerobic conditions, 3-hydroxy-2-formyl benzothiophene was formed by degrading the DBT with the bacterium *Pseudomonas* sp. [4], 1,2-dihydroxy-1,2-dihydrodibenzothiophene and dibenzothiophene 5-oxide were generated by Beijeninckia sp. [33]. Under anaerobic conditions, the biphenyl compound was formed by *Desulfovibrio desulfuricans* M6 [34]. *Corynebacterium* sp. SY1 [35] and *Rhodococcus rhodochrous* IGTS8 were only able to remove sulfur from DBT by converting the DBT into the compound 2-hydroxybiphenyl (2-HBP) [10, 36, 37]. These samples have the ability to remove organic sulfur selectively without degrading the carbon atoms [17]. The biological oxidation of sulfur is primarily catalyzed by two enzyme systems, namely P450 monooxygenases and flavin-containing monooxygenase (FMO). However, in most organisms, the enzymes responsible for oxygenating sulfur (S-oxygenation) have not been clearly identified [5].

A similar behavior was also observed with *C. elegans* (UCP 596), which was able to degrade DBT at the three concentrations studied (0.50 mM and 1 mM) after 24 and 48 h of growth. This showed the metabolites dibenzothiophene 5-oxide, 5,5-dibenzothiophene dioxide, and at the end of the experimental period of 96 h of growth, the compound 2-hydroxybiphenyl was detected. These results suggest the fungus uses the "4S" metabolic pathway

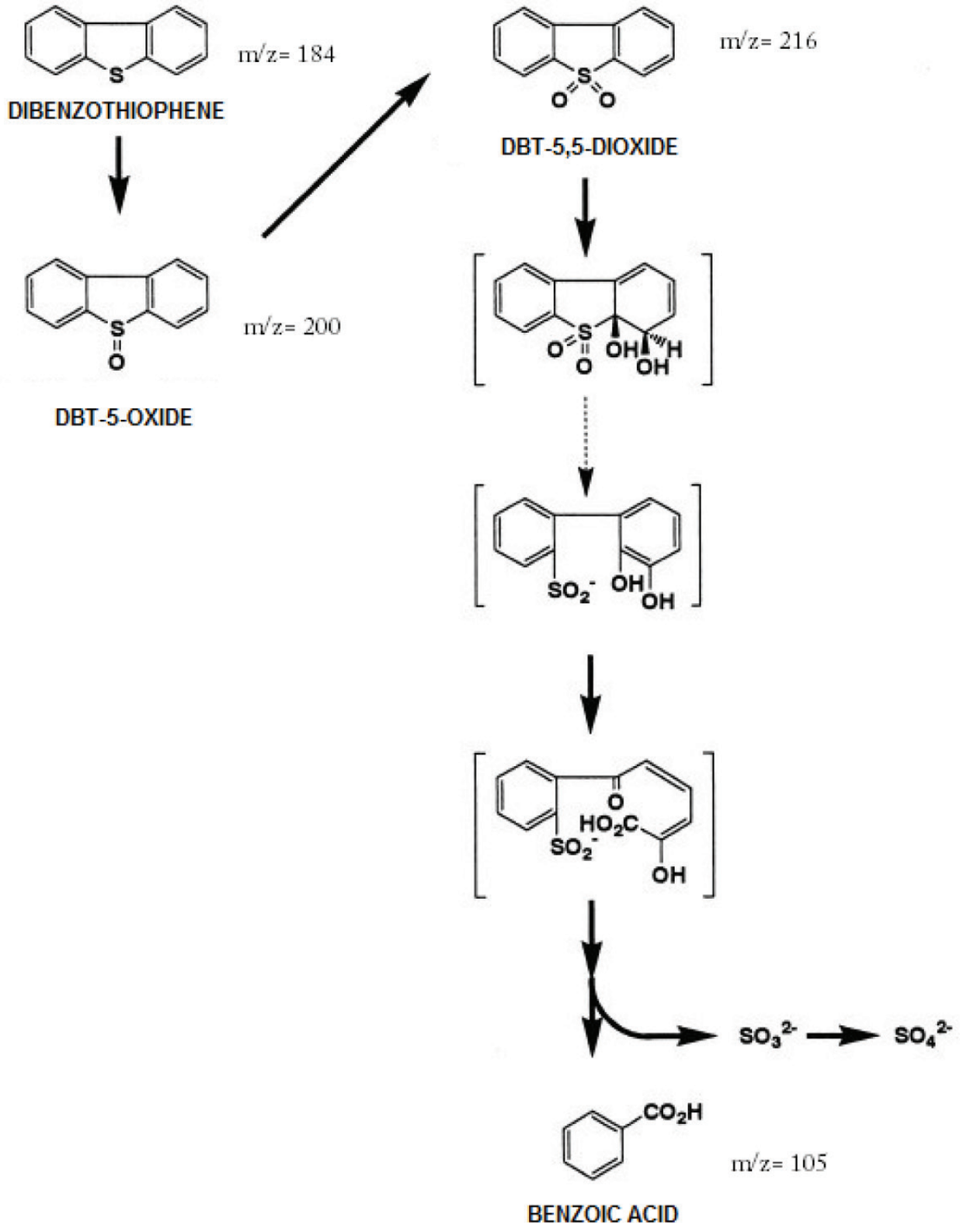

Figure 2. Suggestion for metabolic pathway used by *Cunninghamella elegans* (UCP 596) to remove the sulfur from the DBT inserted into the liquid Sabouraud culture medium, at 28°C, while being shaken at 150 rpm.

Molecular ion (m/z)	Probable compounds formed	Fragments of ions—m/z (relative to intensity %)
184	Dibenzothiophene	185 (13.37), 184 (100), 139.3 (12.14), 45.1 (10.43)
200.2	Dibenzothiophene 5-oxide	201.2 (19.05), 200.2 (100), 172.5 (43.06), 171.5 (73.15), 139.3 (12.42), 86.3 (5.95), 50.2 (14.25)
216.1	Dibenzothiophene 5,5-dioxide	216.1 (100), 187.2 (21.34), 144.3 (16.12), 104.3 (7.98), 50.2 (13.12)
170.2	2-Hydroxybiphenyl	227.3 (5.58), 170.2 (100), 140.2 (16.10), 114.2 (53.73), 85.2 (17.38), 41.2 (13.38)
105.1	Benzoic acid	118.0 (22.67), 105.1 (100), 58.2 (15.49), 45.1 (60.30)

Table 2. Characterization of the mass spectrum of the products from degrading DBT by *Cunninghamella elegans* (UCP 0596).

sulfoxide-sulfone-sulfonate-sulfate. As the specific pathway for removing sulfur atoms present in the DBT due to a progressive oxidative attack in the thiophene groups, this pathway involves a multi-enzyme system with three different activities The first enzyme is a monooxygenase of the DBT, which oxidizes DBT to 5,5-dioxide of DBT. The second enzyme is also a monooxygenase that converts 5,5-dioxide of DBT to 2-hydroxybiphenyl-2-sulfinate. Finally, a lyase that catalyzes the breaking of the C-S link, transforms the 2-hydroxybiphenyl-2-sulfinate into two end products, namely 2-hydroxybiphenyl (HBP) and sulfate. The microorganisms that use this pathway to metabolize DBT manage to remove the potentially toxic atom of the thiophenic compound, in the form of a sulfate compound, with only a slight loss of energy value occurring [16, 17, 20, 26]. Thus, the strains that use the "4S" pathway will be able to constitute a fundamental biological tool in the treatment, on a large scale, of fossil fuels, if biocatalysts in an industrial environment can be obtained [2, 36, 37].

In addition, benzoic acid was detected after 48 h of cell growth. This is a compound present in the Van Afferden or angular dioxygenation pathway that uses the DBT as a carbon source and removes the sulfur in the form of sulfite ion, the final product of which is benzoic acid. The metabolic pathway known as Van Afferden has no great interest in terms of biodesulfurization processes of fossil fuels, since the complete mineralization of the carbonaceous structure occurs, which will necessarily involve reducing the potential chemical energy of the fuels. However, microorganisms that use this metabolic pathway are potentially useful in formulating a mixed microbial inoculum for bioremediation processes of polyaromatic hydrocarbons containing sulfur released into the environment [7, 38, 39].

The results show that *C. elegans* (UCP 596) was able to degrade about 70% of DBT in 24 h of growth in Sabouraud culture medium treated with 0.50 mM of DBT, and that after 96 h of culture, the reduction was 97% of DBT from the initial content. On the other hand, the sample of *C. elegans* grown on Sabouraud treated with 1.0 mM of DBT, showed a reduction of 98.1%, after 96 h of growth, thus reducing the initial concentration to 0.019 mM of DBT.

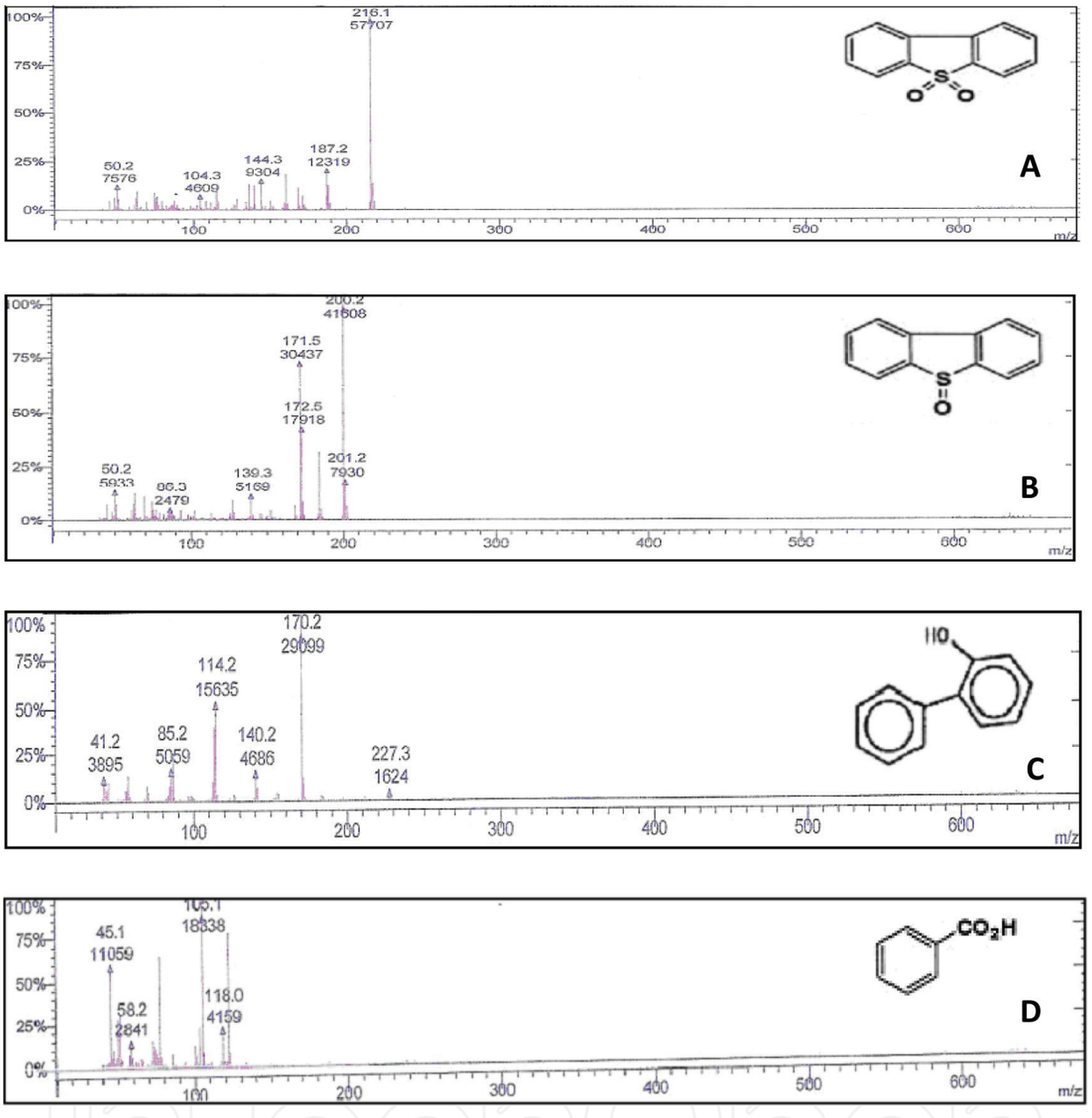

Figure 3. Mass spectrogram of dibenzothiophene 5-oxide (A), dibenzothiophene 5,5-dioxide (B), 2-hydroxybiphenyl (C), and benzoic acid (D). Metabolites formed on degrading dibenzothiophene by *Cunninghamella elegans* after 48 h of growth.

According to Schlenk et al. [13], the filamentous fungus *C. elegans* ATCC-36112, metabolized approximately 98% of dibenzothiophene at a concentration of 1.8 mg/mL for dibenzothiophene 5-oxide (86% of total metabolites) and dibenzothiophene 5-dioxide (14% of total metabolites), after incubation for 24 h. The fungus *Pleurotus ostreatus* grown in culture medium containing DBT metabolized about 84% of organosulfur in 21 days of growth. The results were analyzed by HPLC, and the main compounds formed were dibenzothiophene 5-oxide and dibenzothiophene 5,5-dioxide. The lignolitic mechanism of the fungus *P. ostreatus* may be

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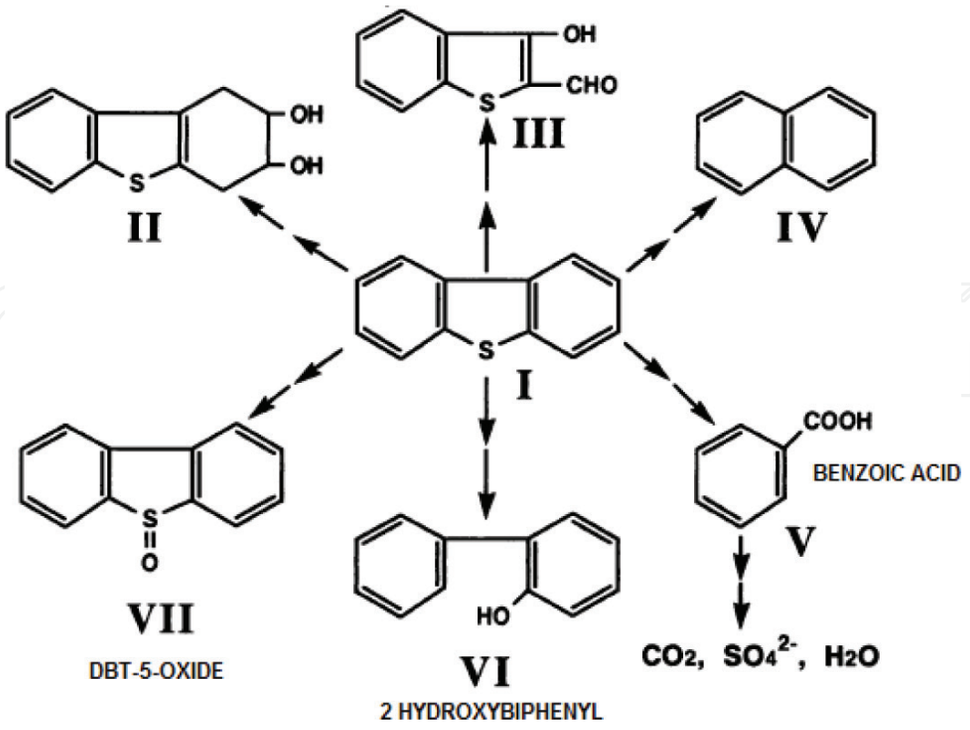

Figure 4. Pathways proposed for biodegrading dibenzothiophene by *Cunninghamella elegans*: (I) dibenzothiophene, (II) 1,2-dihydroxi-1,2-dihydrodibenzothiophene, (III) 3-hydroxy-2-formyl benzothiophene, (IV) biphenyl, (V) benzoic acid, (VI) 2-hydroxybiphenyl and (VII) dibenzothiophene 5-oxide.

involved in the metabolism of DBT [15, 40]. *Arthrobacter* sp. used the angular deoxygenating pathway for metabolizing 5,5-dioxide of DBT, a compound used as a source of carbon [41].

The data presented indicate the potential of *C. elegans* UCP 0596 using DBT as the sole source of sulfur and the greater efficiency of the fungus pathway at removing sulfur during the catabolism and that the products formed are of great importance. That activity pattern, which is shown by *C. elegans* UCP 0596 forming different biomolecules, gives information on their cellular characteristics, which undoubtedly express the potential abilities of the organism.

4. Conclusion

C. elegans UCP-596 has the ability to metabolize dibenzothiophene by an oxidation process which formed dibenzothiophene 5-oxide, dibenzothiophene 5,5-dioxide, and 2-hydroxybiphenyl metabolites. The greater efficiency of the fungus at removing sulfur suggests the use of the "4S" metabolic pathway. It can be considered for the upstream removal of sulfur.

The activity pattern of DBT degradation by *C. elegans* gives potential to benzoic acid production as the end of cellular catabolism. The fungus strain is able to desulfurize DBT in the use of angular dioxygenation pathway, as a second metabolic pathway. Its selective sulfur removal makes it proper for the industrial use of *C. elegans* UCP-596 strain.

Acknowledgements

This work was supported by Coordination Unit for Improving the Qualifications of Higher Education staff (CAPES), Foundation for Supporting Science and Technology of the State of Pernambuco (FACEPE), and National Council for Scientific and Technological Development (CNPq). The authors are grateful to the Catholic University of Pernambuco (Recife-PE, Brazil) for the use of its laboratories and the technician Severino Humberto de Almeida for his assistance.

Author details

Patrícia Mendes de Souza[1,5], Thayse Alves de Lima e Silva[1,5],
Marcos Antonio Barbosa Lima[2], Luciana de Oliveira Franco[2], Manfred Schwartz[3],
Paulo Henrique da Silva[5], Lúcia Roberta Barbosa[4], Aline Elesbão do Nascimento[5],
Kaoru Okada[5] and Galba Maria de Campos-Takaki[5]*

*Address all correspondence to: galba_takaki@yahoo.com.br

1 Catholic University of Pernambuco – UNICAP, Recife, PE, Brazil

2 Department of Biology, Federal Rural University of Pernambuco, Recife, PE, Brazil

3 SI Group Crios Resinas S.A., Rio Claro, SP, Brazil

4 Foundation of Higher Education in Olinda – FUNESO, Olinda, PE, Brazil

5 Nucleus for Research in Environmental Sciences and Biotechnology, Catholic University of Pernambuco, Recife, PE, Brazil

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