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POTENCIAL BIOTECNOLÓGICO DE *Pseudomonas fluorescens* UCP 1514 NO PROCESSO DEGRADATIVO DO DIBENZOTIOFENO

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“Feliz o homem que acha sabedoria, e o homem que adquire conhecimento; porque melhor é o lucro que ela dá do que o da prata, e melhor sua renda do que o ouro mais fino”.

(Provérbios 3:13-14)

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RESUMO

O óleo bruto e seus destilados contêm quantidades significantes de compostos organossulfurados de baixo peso molecular, tais como tióis cicloalquilas e compostos aromáticos heterocíclicos baseados em tionefóis. Este último grupo de hidrocarbonetos aromáticos policíclicos sulfurados inclui benzotiofeno, dibenzotiofeno e seus derivados. Estes compostos têm sido objetos de interesse de estudos há décadas, pelo fato de constituírem a maior classe de contaminantes ambientais encontrados na água e no solo. Com o objetivo de consertar as conseqüências desta contaminação, tais como chuva ácida e poluição do ar, causadas pelo dióxido de enxofre liberado da combustão dos óleos, mais regulamentações na quantidade de enxofre no petróleo estão sendo estabelecidas. A biodessulfurização tem atraído a atenção dos pesquisadores pela sua aplicação na dessulfurização do petróleo, devido às condições do meio, como baixo consumo de energia e baixa emissão de dióxido de enxofre. Devido a esses fatores, a biodessulfurização, que opera em condições ambiente de temperatura e pressão, possui um grande potencial como uma técnica bastante promissora em alternativa à hidrodessulfurização. Neste trabalho foi estudada a cinética de crescimento de *Pseudomonas fluorescens* UCP 1514, que é uma linhagem selvagem do solo, em culturas em batelada contendo dibenzotiofeno, e seu potencial na biodessulfurização, para utilização na redução de enxofre contido nos combustíveis fósseis, de acordo com as regulamentações ambientais. O microrganismo foi capaz de metabolizar o composto e foram também identificadas através da amplificação do DNA, colônias mutantes fisiológicas, que cresceram na presença do dibenzotiofeno. Os resultados mostraram um decréscimo de 73% na concentração do composto, indicando que *Pseudomonas fluorescens,* seletivamente, remove enxofre do dibenzotiofeno para formar bifenil, indicando que este microrganismo oferece grande potencial na biodegradação de compostos sulfurados, podendo ser utilizado como catalisador biológico na desssulfurização e mineralização desses compostos, consequentemente, minimizando os danos ambientais causados pela poluição dessas substâncias.

ABSTRACT

Crude oil and its distillates contain significant amounts of low-molecular-mass organosulphur compounds such as alkyland cycloalkyl thiols, alkyl- and arylthioethers and aromatic heterocycles based on thiophene. This last group of polycyclic aromatic sulfurated hydrocarbons includes thiophene itself, benzothiophene, dibenzothiophene, and their alkylated derivatives. These compounds have been of concern for decades because they constitute a major class of ubiquitous environmental contaminants found in both air and sea areas. In order to mitigate the consequences of this contamination, such as acid rain and air pollution caused by sulphur dioxide released from the combustion of oils, more and more regulations on sulphur content in petroleum are being established. Biodesulfurization has attracted attention owing to its application to the desulfurization of petroleum due to its mild conditions, lower energy consumption and lower emission of sulphur dioxide. Thus biodesulfurization, which operates under room temperature and pressure conditions, is expected to be a complementary as well as promising alternative to hydrodesulfurization. In this work, we studied the growth kinetics of a bacterium of the strain *Pseudomonas fluorescens* UCP 1514, which is indigenous in soil, in batch cultures containing dibenzothiophene, a recalcitrant component of fossil fuels and its potential on the desulfurization, in order to use it for reducing the sulfur content of diesel oil in compliance with environmental regulations. The microorganism was capable to metabolize dibenzothiophene and we also identified the physiological mutant colonies that growing on dibenzothiopnhene by DNA amplification with polymerase chain reaction technique and the specific primer BOX.The results showed a decrease of 73% in dibenzothiophene content, indicating that *Pseudomonas fluorescens* selectively removes sulfur from dibenzothiophene to form biphenyl, indicating that this bacterium shows promising potential for decreasing the sulfur content of fossil fuels, can be used as biological catalyst, on desulfurization and mineralization these compounds, minimizing the problems caused by pollution of this substances.

SUMÁRIO

LISTA DE FIGURAS E TABELAS

CAPÍTULO I

CAPÍTULO II

CAPÍTULO III

LISTA DE ABREVIATURAS

BRS: Bactérias redutoras de sulfato

BDS: Biodessulfururização

DBT: Dibenzotiofeno

GLP: Gás liquefeito de petróleo

HDS: Hidrodessulfurização

4S: Via do 4 S (Sulfoxi-Sulfona-Sulfonato-Sulfato)

CAPÍTULO I

REVISÃO BIBLIOGRÁFICA

1. INTRODUÇÃO

A poluição ambiental e a chuva ácida causadas pela liberação de dióxido de enxofre na combustão do enxofre contido nos combustíveis fósseis conduziram a regulamentos ambientais estritos para produção de baixas concentrações desses compostos orgânicos sulfurados. Considerando a utilização crescente destes compostos, devido em grande parte às necessidades dos países industrializados, a emissão do enxofre para a atmosfera constitui, assim, um dos problemas ambientais da atualidade (SWATY, 2005).

A existência da técnica de hidrodessulfurização aplicada em refinarias de petróleo para remover o enxofre envolve tratamento catalítico do combustível e alta temperatura e pressão. Mas esta técnica tem demonstrado não ser muito eficiente, pois além de utilizar métodos físico-químicos muito caros, não remove completamente os compostos policíclicos sulfurados. Uma estratégia para redução da quantidade de enxofre é expor estes substratos a microrganismos capazes de quebrar a ligação carbono-enxofre, e desse modo, liberar o enxofre na forma inorgânica. Este processo de dessulfurização microbiológica ou biodessulfurização pode ser associado com a hidrodessulfurização e superar os problemas técnicos e econômicos, reduzindo os custos e trazendo benefícios, por produzir menos gases que provocam o efeito estufa (VAN HAMME et al., 2003).

Os estudos de biodessulfurização tiveram início nas décadas de 50 e 60, no entanto, apenas nos últimos anos, com o advento da biotecnologia, esta área passou a apresentar desenvolvimento significativo. O dibenzotiofeno (DBT) é um composto organossulfurado heterocíclico, e como outros compostos análogos com substituintes alquil, sendo utilizados quer como fonte de carbono e energia principal ou secundária (co-substrato), por serem considerados compostos recalcitrantes presentes nos combustíveis fósseis. O DBT é, no entanto, geralmente designado como composto modelo no processo de biodessulfurização. (MONTICELLO,1985).

Kodama et al. (1973) foram os primeiros a reportar a conversão aeróbica do DBT. A transformação do DBT resulta na clivagem de um dos anéis aromáticos, enquanto que o enxofre não é liberado, sendo denominado como a via de “Kodama”. Outro estudo realizado com *Brevibacterium* sp. apresenta uma via utilizando o DBT como única fonte de carbono, enxofre e energia. Mas devido à degradação das ligações de carbono (C-C) o valor

calórico é alterado, conseqüentemente esta via não é desejável no processo de biodessulfurização.

A primeira linhagem de bactéria seletiva do enxofre *Rhodococcus erythropolis* foi isolada por Kilbane et al. (1989). Esta espécie é capaz de conduzir um passo importante na oxidação seletiva do heteroátomo de enxofre, enquanto a estrutura do carbono não é metabolizada. Trata-se da via sulfóxido-sulfona-sulfonato-sulfato"4S" (KAYSER et al., 1993). Os microrganismos que utilizam esta via para metabolizar o DBT conseguem assim, que o átomo potencialmente tóxico seja retirado do composto tiofênico, sob a forma de um composto tratável (sulfato), constituindo uma ferramenta biológica fundamental no tratamento em larga escala dos combustíveis fósseis, caso se consigam obter biocatalisadores de elevada estabilidade em ambiente industrial (WANG; KRAWIEC, 1994).

Estudos de biologia molecular realizados com *Rhodococcus* e *Pseudomonas* sp. têm permitido conhecer a sequenciação dos fragmentos de DNA que possuem os genes da via "4S"(ALVES et al., 1999).

O processo microbiológico da dessulfurização descrito pode ser usado reduzindo a quantidade de enxofre contida no óleo diesel, particularmente, depois do processo de hidrodessulfurização, o que ajudaria na regulamentação dos padrões dos níveis de enxofre no óleo diesel (LABANA,. et al., 2005; PAZ, et al., 2005).

A utilização de bactérias capazes de degradar o DBT e outros derivados de petróleo tem sido objeto de vários trabalhos de pesquisa. A biodessulfurização está baseada na via "4S", na qual o DBT não é egrada, e sim, tranformado em 2-HBP, o que resulta em nenhuma perda calorífica do combustível e, atualmente, vários gêneros bacterianos com este potencial estão sendo identificados (MOHEBALI et al., 2007).

A espécie *Pseudomonas fluorescens* é uma bactéria considerada cosmopolita, Gram-negativa, aeróbica, não patogênica, que apresenta flagelos em sua morfologia e seu metabolismo possui uma vasta versatilidade. Estudos realizados com esta espécie demonstraram o seu potencial na produção de biossurfactantes, formação de biofilmes e degradação de petróleo e seus derivados (BARATHI et al., 2001).

Considerando os impactos causados por DBT no ambiente, de acordo com os dados relatados pela literatura (VAN HAMME et al., 2006), este trabalho objetivou investigar o

comportamento bioquímico e fisiológico de *Pseudomonas fluorescens* na transformação do dibenzotiofeno-DBT, avaliando o potencial no processo de biodessulfurização, na produção de metabólitos de fácil aproveitamento no meio ambiente e uma promissora aplicação industrial no tratamento dos combustíveis das refinarias de petróleo.

2. OBJETIVOS

2.1. Geral

Considerando a importância do tratamento de compostos recalcitrantes derivados de petróleo, neste trabalho foi avaliado o comportamento bioquímico e fisiológico de *Pseudomonas fluorescens* no processo de degradação do dibenzotiofeno.

2.2. Específicos

- Avaliar a influência do dibenzotiofeno no crescimento de *Pseudomonas fluorescens.*

- Realizar a cinética de crescimento e o comportamento bioquímico e fisiológico de *Pseudomonas fluorescens* no processo de degradação do dibenzotiofeno.

- Investigar a eficiência *Pseudomonas fluorescens* no processo de biodessulfurização e biodegradação do dibenzotiofeno, analisando os subprodutos formados.

3. REVISÃO BIBLIOGRÁFICA

3.1. Petróleo e derivados

3.1.1. História e origem do petróleo

O petróleo e o carvão são importantes combustíveis fósseis com uma composição complexa em que se podem considerar quatro famílias de compostos: hidrocarbonetos alifáticos, cíclicos, aromáticos e moléculas contendo átomos de azoto, enxofre ou oxigênio na sua estrutura (Tabela 1). Os compostos orgânicos contendo enxofre (S) constituem uma pequena, mas importante fração desses combustíveis e devido à sua difícil biodegradabilidade são considerados compostos recalcitrantes (KROPP et al., 1997).

O petróleo é composto de hidrocarbonetos em seus três estados e é considerado a principal fonte de energia mundial. Contém pequenas quantidades de compostos de enxofre, oxigênio, nitrogênio. Na antiguidade, era usado para fins medicinais ou para lubrificação, sendo conhecido com os nomes de óleo de pedra, óleo mineral e óleo de nafta. Atribuíam-se ao petróleo propriedades laxantes, cicatrizantes e anti-sépticas. Os resíduos orgânicos, de bactérias, de produtos nitrogenados e sulfurados no petróleo indicam que ele é o resultado de uma transformação da matéria orgânica acumulada por milhões de anos nas profundezas dos oceanos, dos mares e dos solos, sob pressão das camadas de sedimentos que foram se depositando e formando rochas sedimentares (AECIPE, 2002).

O conjunto dos produtos provenientes desta degradação, hidrocarbonetos e compostos voláteis, misturados aos sedimentos e aos resíduos orgânicos, contido na rocha-mãe; a partir daí o petróleo é expulso sob efeito da compactação provocada pela sedimentação, migrando para impregnar areias ou rochas mais porosas e mais permeáveis, tais como arenitos ou calcários (SACCHETTA, 2003).

Os produtos derivados do petróleo como os gases (metano, etano, propano e butano) apresentam importância pelo seu emprego como fontes de energia na sociedade atual. A gasolina, o querosene, o diesel, os óleos, as graxas e as ceras, sem deixar de comentar o asfalto, o produto final do processo de refino, aplica-se em inúmeros processos de desenvolvimento atual (SACCHETTA, 2003). Todos estes produtos, de alguma forma, contribuem para a economia mundial.

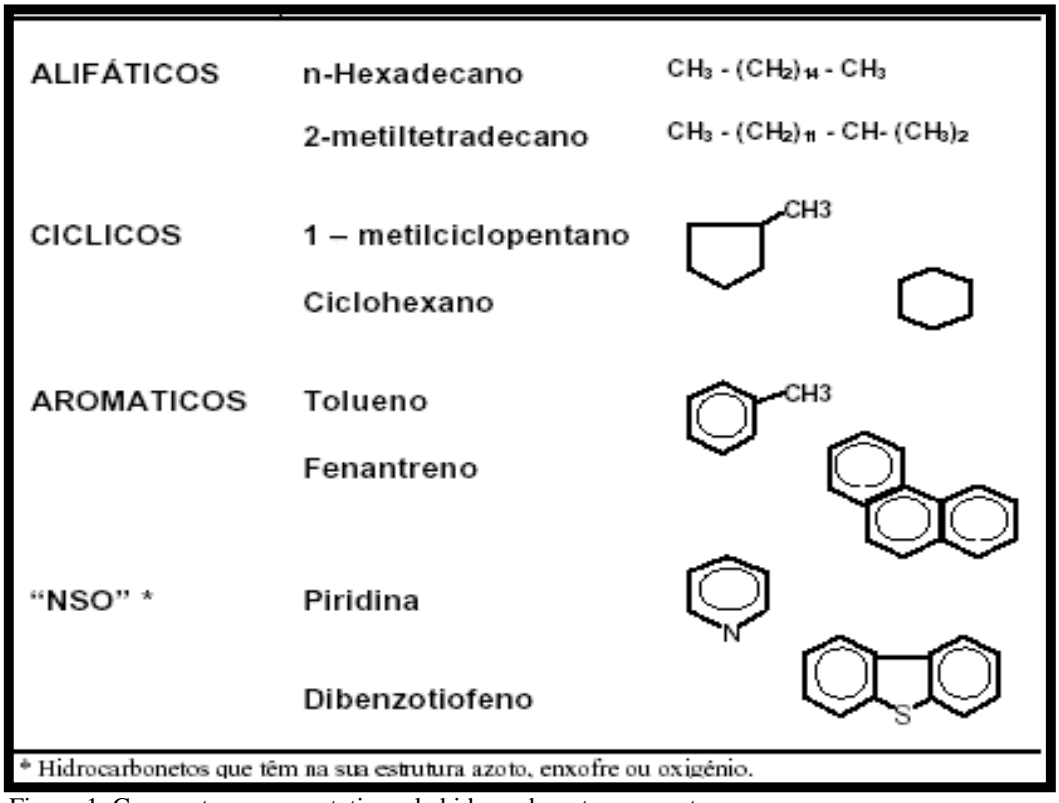

Figura 1. Compostos representativos de hidrocarbonetos presentes nos combustíveis fósseis. Fonte: ALVES, et al., 1999

3.1.2. Processo de refino

A função das refinarias consiste em dividir o óleo cru em frações (grupos) delimitadas pelo ponto de ebulição de seus componentes, e em seguida reduzir essas frações a seus diversos produtos. Quando possível, os processos de refino são adaptados à demanda dos consumidores. Assim é que no final do século XIX, quando o querosene de iluminação era muito utilizado, as refinarias dos Estados Unidos extraíam do óleo cru até setenta por cento de querosene. Depois, quando a gasolina passou a ser o subproduto mais procurado, começou a ser retirada do óleo cru nessa porcentagem. Mais tarde, o querosene voltou a encontrar larga aplicação como combustível para aviões a jato. As refinarias localizam-se muitas vezes junto às fontes produtoras, mas também podem situar-se em pontos de transbordo ou perto de mercados de consumo, que oferecem a vantagem da redução de custo, pois é mais econômico transportar petróleo bruto por oleodutos do que, por outros meios, quantidades menores de seus derivados (PEREIRA JUNIOR & LOUVISSE, 2000).

Na refinaria, o óleo cru e os produtos semifinais e finais são continuamente aquecidos, resfriados, postos em contato com matérias não orgânicas, vaporizados, condensados, agitados, destilados sob pressão e submetidos à polimerização (união de várias moléculas idênticas para formar uma nova molécula mais pesada) sem intervenção humana. Os processos de refino podem ser divididos em três classes: separação física, alteração química e purificação.

A primeira etapa do processo de refino de petróleo é a destilação primária, através da qual são extraídas do petróleo as principais frações que dão origem à gasolina e ao óleo diesel, toda a nafta, os solventes e querosenes (de iluminação e aviação), além de parte do GLP (gás de cozinha). Em seguida, o resíduo da destilação primária é processado na destilação a vácuo, onde é extraída do petróleo mais uma parcela de diesel, além de frações de um produto pesado chamado de gasóleo, que pode ser destinado à produção de lubrificantes ou a processo mais sofisticados, como o craqueamento catalítico, onde é transformado em GLP, gasolina e óleo diesel; o resíduo da destilação a vácuo pode ser usado como asfalto ou destinado à produção de óleo combustível. Uma série de produtos é obtida, tendo em vista as necessidades dos consumidores e produtos diversos (PEREIRA JUNIOR & LOUVISSE, 2000).

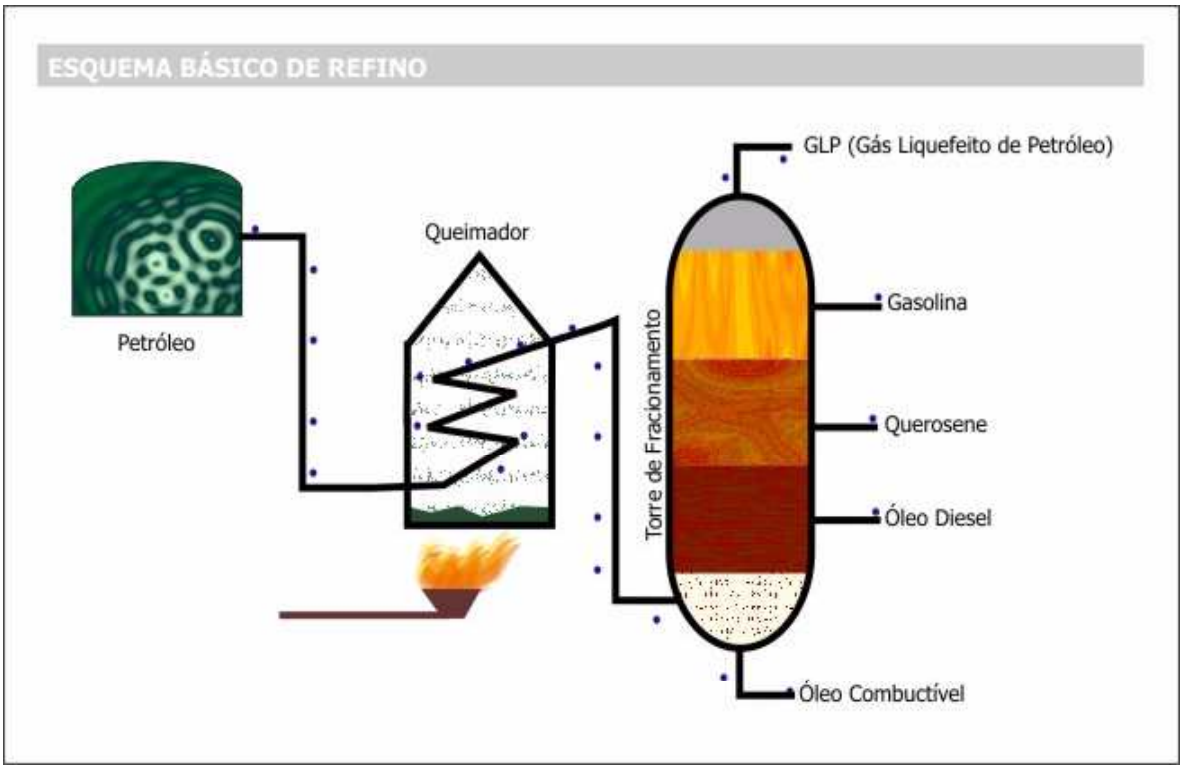

Figura 2. Processo de refino do petróleo. (Fonte: www.cepa.if.usp.br/energia)

3.1.3. Efluentes industriais petroquímicos

Os resíduos industriais petroquímicos apresentam complexa mistura de compostos divididos em quatro famílias: hidrocarbonetos alifáticos, cíclicos, aromáticos e compostos que apresentam enxofre em sua estrutura, denominado genericamente como organossulfurados (ALVES et al., 1999). Os compostos organossulfurados reperesentam uma pequena parcela nos efluentes de refinarias, que em geral, apresentam grande variedade de compostos recalcitrantes (benzotiofeno, dimetilsulfóxido, fenilsulfeto, sulfonas etc). Embora em quantidades consideradas pequenas, são extremamente nocivos ao homem e ao ambiente, já que sua presença pode causar irritação das mucosas, espasmos musculares e dos brônquios, além de provocar corrosão nos equipamentos da refinaria e chuva ácida (DENOME et al., 1993; ALVES et al., 1999).

Existe uma variedade de compostos orgânicos sulfurados que se encontram na combustão do petróleo, entre eles, o dibenzotiofeno (DBT), um composto recalcitrante encontrado nos destilados médios (óleo diesel, lubrificantes finos etc) e o estudo do seu tratamento tem sido considerado modelo no tratamento de combustíveis fósseis (RHEE et al., 1998).

3.2. Compostos organossulfurados

Emissões antropogênicas de enxofre possuem um papel crucial em três problemas ambientais importantes: poluição do ar, chuva ácida e mudança climática global. As emissões globais aumentaram muito até o final dos anos 80 (LEFOHN et al., 1999). E as estimativas globais de 1990 a 2000, mostraram uma relativa estabilidade, com um declínio moderado durante toda a década (OLIVIER & BERDOSWSKI, 2001; SMITH et al., 2001).

O teor de enxofre nos combustíveis está sendo continuamente reduzido por regulamentações, a níveis cada vez mais baixos. Europa e Estados Unidos, atualmente, exigem um teor máximo de 50ppm de enxofre, na gasolina e no óleo diesel e este nível será reduzido para menos de 10ppm para o ano de 2010 (EN 228-590/1999; KOCAL et al., 2002; ALI et al., 2006).

Os compostos organossulfurados são encontrados nos combustíveis como óleo diesel, gás de cozinha, gasolina e alguns lubrificantes de motores, que apresentam uma

certa quantidade de enxofre. Nos dias atuais, seu uso está sendo controlado pelos órgãos de defesa ambiental de diversos países desenvolvidos.

Os compostos orgânicos que apresentam enxofre nos combustíveis fósseis formam uma fração embora pequena, muito importante devido a sua baixa degradabilidade. O petróleo bruto possui entre 0,04 - 5% de enxofre, e, quanto mais denso maior é essa fração (KROPP et al., 1997). A partir de então, devem diminuir suas concentrações com a finalidade de minimizar a poluição do ar, do solo e da água, e os danos causados pela chuva ácida (FURUYA et al., 2003).

No Brasil, a legislação ambiental que é rica em regulamentações quanto à disposição e tratamento de resíduos recalcitrantes, busca implementar leis que visem sanar possíveis transtornos, que possam ocorrer caso não haja um controle rígido dos resíduos gerados na queima dos combustíveis fósseis, principalmente os produtos que contaminam o ar, o solo e a água. Para cada ecossistema avaliado há em vigência, uma gama de regulamentações no manejo destes, que buscam não atrapalhar o desenvolvimento sócio-econômico do país e sim, buscar desenvolver sem causar sérios danos ao meio ambiente. Entretanto, os constantes esforços da população científica buscam alternativas de combustíveis e produtos menos impactantes ao meio ambiente, tais como o biodiesel, e métodos de tratamento de baixo custo e alta eficiência (PAZ, et al., 2005).

3.3. Tratamento de compostos organossulfurados

O tratamento convencional de alguns compostos organossulfurados dos combustíveis fósseis como o dibenzotiofeno (DBT) (Fig. 2.) é feito através da técnica de hidrodessulfurização, empregada nas refinarias, a qual requer emprego de altas temperaturas e gastos elevados de energia, e assim, o mesmo não remove efetivamente os compostos sulfurados policíclicos (FOLSOM et al., 1999).

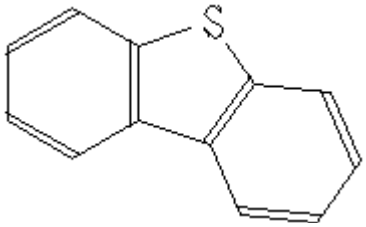

Figura 3. Estrutura química do dibenzotiofeno
Fonte: Kropp et al., 1997

3.3.1. Dessulfurização do diesel

Enquanto o desafio da hidrodessulfurização é conseguir uma alta seletividade na hidrogenação do enxofre, a principal questão na hidrodessulfurização do diesel é a baixa reatividade das muitas espécies de compostos aromáticos com enxofre. A questão já foi discutida nos anos 80 (NAG et al., 1979; HOVALLA et al., 1980) e revisada por Girgis e Gates (1991).

Existem muitos trabalhos que estudam uma variedade de alternativas possíveis para melhorar o processo de HDS, e dentre eles, está descrito o método oxidativo. Enquanto a HDS tenta reduzir o enxofre para formar H_2S, o método de oxidação objetiva promover uma reação na direção oposta, para formar espécies de enxofre oxidadas (ITO & VEEN, 2006).

A dessulfurização oxidativa parece oferecer algumas vantagens, comparadas com a HDS, e dentre elas, o uso de condições amenas de reação (ITO & VEEN, 2006).

Uma opção muito atrativa é utilizar um sistema biológico para fazer a oxidação química do enxofre. A reação ocorre na presença de água e O_2, à temperatura e pressão ambiente (BORON et al., 1999).

Existem algumas companhias que têm investigado bactérias que metabolizam benzotiofeno e dibenzotiofeno, além do desenvolvimento de suas atividades, para aplicação em processos de biodessulfurização (SONG et al., 2000; KLEIJAN et al., 2003). Aspectos importantes devem ser levados em consideração com relação à este processo, tais como a alta atividade microbiana e as reações competitivas causadas por outras bactérias. Um processo biológico de H_2S bem-sucedido tem uma ótima vantagem, pois apenas o tipo alvo de bactéria parece sobreviver no ambiente altamente contaminado com H_2S, convertendo a enxofre elementar eficientemente. Nesse aspecto, a dessulfurização do diesel seria um esforço adicional para promover o trabalho da bactéria em questão. Atualmente, muitos trabalhos têm apresentado o desenvolvimento de novas bactérias para a dessulfurização do petróleo (MINGFAN et al., 2003; MAGHSOUDI et al., 2004).

3.3.2. Hidrodessulfurização (HDS) convencional

A hidrodessulfurização é uma tecnologia físico-química, estabelecida para remover organicamente ligações de enxofre a níveis epecíficos (SPEIGHT, 1981). Como a dessulfurização de compostos organossulfurados apresenta um ponto de ebulição alto (de 250°C a 350°C) ela é de importância particular (SCHULZ et al., 1999). A remoção da maior parte do enxofre presente no gasóleo é convencionalmente executada por hidrotratamento com um fluxo descendente de gás hidrogênio e hidrocarboneto em um catalisador. Em presença do catalisador, o gás hidrogênio reage com os compostos sulfurados para produzir hidrogênio-sulfídeo gasoso. As condições típicas de HDS são temperaturas entre 200 e 350°C e pressões de 5 a 10 MPa, dependendo da severidade da dessulfurização exigida (MARCELIS, 2002).

Infelizmente, a técnica convencional não é satisfatória para conhecer o futuro especificado dos níveis de dessulfurização profunda. Aspectos importantes que possuem um papel para alcançar estes níveis são a aplicação de um catalisador moderno digital e inovações das configurações no processo de HDS (MARCELIS, 2002).

3.3.3.Biorremediação de compostos organossulfurados

Todos os organismos requerem enxofre para a síntese de proteína e cofatores essenciais, podendo estes ser assimilados a partir de fontes inorgânicas como os sulfatos e os tiossulfatos, ou de fontes orgânicas, como ésteres sulfatos e sulfonados (COPPÉE et al., 2001).

Segundo Tortora *et al*. (2003), algumas bactérias dos gêneros *Pseudomonas, Proteus, Campylobacter e Salmonella* são capazes de reduzir o enxofre elementar (S°), bem como outros compostos sulfurados como tiosulfato, sulfeto e dimetilsulfóxido, pois apresentam uma propriedade muito comum na variedade heterotrófica, sendo geralmente aeróbias facultativas.

Os microrganismos capazes de degradar compostos sulfurados são diversos quanto às suas características morfológicas. Os representantes do gênero *Bacillus,* são capazes de reduzir o enxofre elementar (S°), tiosulfato, sulfeto entre outros compostos sulfurados, devido a sua característica heterotrófica, mesmo que a maioria seja anaeróbia estrita. Os gêneros *Desulfonema e Desulfovibrio*, vários autores encontraram redutores de sulfato sob

condições temporária de aerobiose na interface oxi-anóxica de sedimentos (de até 5% de oxigênio), onde cresceriam pobremente, caracterizando-se por apresentar mecanismos de redução das concentrações deste, assim como repostas comportamentais para se proteger, como migração, formação de agregados e de bandas na região mais anóxica do gradiente oxi-anóxico do sedimento ou das condições artificiais de cultura (SASS et al. 2002).

As bactérias redutoras de enxofre (SRB), constituem um grupo de procariotos bastante diversificado, que contribui para a manutenção de processos essenciais no ambiente, tais como os de regulação da ciclagem da matéria orgânica e até a metilação do mercúrio (CASTRO et al. 2000).

3.3.3.1. Bactérias redutoras de sulfato (BRS)

As bactérias redutoras de sulfato (BRS) são microrganismos que realizam a redução desassimilativa do íon sulfato, na qual este íon atua como agente oxidante para o metabolismo da matéria orgânica. Nesse processo apenas uma pequena parcela do enxofre reduzido é assimilada pelos microrganismos, sendo a maior parte excretada na forma de íon sulfeto normalmente hidrolisado a H_2S livre (POSTGATE, 1984).

Em sua maioria as BRS são Gram-negativas e mesofílicas, com temperatura ótima de crescimento na faixa de 34 a 37ºC e com tolerância máxima de 42 a 45ºC. Linhagens termofílicas, apresentando crescimento ótimo na faixa de 50 a 70 ºC, podem ser encontradas também.

O crescimento das BRS mesofílicas, a 30°C, é normalmente lento podendo levar semanas de acordo com a espécie**.** Por outro lado, as BRS termofílicas crescem rapidamente em torno de 12 a 18 h, a 55°C. Em geral, no crescimento das BRS, a temperatura de incubação normalmente utilizada é de 30ºC, enquanto que a faixa de pH mais empregada é de 7,2 a 7,6 (SÉRVULO, 2001).

O metabolismo oxidativo das BRS é conduzido em ambientes cujo potencial de oxi-redução se encontre na faixa de –150 a –200 mV, uma vez que esses microrganismos não necessitam possuir co-fatores da cadeia de transporte de elétrons cujas formas estáveis só são encontradas em valores de potencial redox positivos. É necessário então, em algumas situações, que seja adicionado um agente redutor, como o Na_2S, até que o crescimento vigoroso da cultura seja estabelecido e assim as próprias bactérias gerem o H_2S suficiente

para manter o potencial de oxi-redução baixo (POSTAGATE, 1984; OLIVEIRA et al., 2004).

3.3.4. Dessulfurização microbiana (Biodessulfurização BDS)

O tratamento de compostos organossulrados como o dibenzotiofeno (DBT), pode ocorrer físico-quimicamente, através da hidrodesulfurização, ou biologicamente, pela biodessulfurização, que em geral, acontece através de microrganismos capazes de utilizar o enxofre presente no composto (KROPP et al., 1997; BOTARI et al., 2001).

Devido aos altos custos e emissões atmosféricas da HDS, a dessulfurização biocatalítica tem sido considerada como uma alternativa em potencial para os processos de profunda HDS (MOHEBALI et al., 2007).

Em média, há 20 anos atrás a pesquisa na biodessulfurização tinha como alvo o carvão, com interesse na remoção do enxofre orgânico e inorgânico, pelos microrganismos; no entanto, nos anos recentes, os estudos com o carvão se tornaram menos comuns (BAEK et al., 2002; DEMIRBAS et al., 2004). O tratamento microbiano do enxofre inorgânico do carvão tem progredido a níveis de testes de escala industrial, mas a remoção do enxofre orgânico continua a ser um problema (CARA et al., 2005). As dificuldades ao acesso do enxofre no carvão, quando em comparação com o petróleo, combinado com regulamentações ambientais para níveis de enxofre cada vez menores, tem resultado em mais pesquisas na biodesulfurização, tendo como maior foco, o enxofre orgânico no petróleo (SWATY et al., 2005).

Petróleo contém uma mistura complexa de estruturas químicas, mas os compostos organossulfurados de maior interesse para biodessulfurização são benzotiofenos e dibenzotiofenos (DBTs), primariamente devido à recalcitrância relativa deles para remoção pelos processos da refinaria, especificamente, hidrodessulfurização. (KILBANE ET et al., 2004).

DBT tem se tornado o composto modelo para a pesquisa na biodessulfurização. Numerosas culturas microbianas, incluindo bactérias Gram-positivas e Gram-negativas e leveduras, têm sido isoladas, baseado em suas habilidades em utilizar DBT (ou moléculas relacionadas) como única fonte de enxofre.

Muito mais é conhecido sobre a via de metabolismo do DBT por microrganismos aeróbios e a via para dessulfurização oxidativa do DBT mais conhecida é a do 4S.

Um processo de escala industrial para biodessulfurização do petróleo usando microrganismos aeróbios ainda não foi apresentada, mas através de um entendimento melhorado da bioquímica e genética da via de dessulfurização, antecipadamente, já se pode dizer que, biocatalizadores com atividades mais eficientes para aplicação industrial, serão desenvolvidos (KILBANE, 2006).

3.3.4.1.Vias Metabólicas Degradativas do DBT

- **Via de Kodama (1973)**

Em 1973, Kodama e seus colaboradores, estudando duas espécies de *Pseudomonas* spp. constataram que o DBT era parcialmente degradado através de sucessivas oxidações por um mecanismo semelhante ao da degradação do naftaleno (DENOME et al., 1993) (Fig. 3). A dihidroxilação de um dos anéis aromáticos do DBT conduz à destruição desse anel, obtendo-se como produto final o 3- hidroxi-2-formilo-benzotiofeno no qual persiste o núcleo tiofênico. Esta é a via utilizada pela maioria das bactérias estudadas que atacam o DBT (KILBANE & JACKOWSKY, 1992). Na via de Kodama, o ataque ao anel benzênico ocorre nas posições 2 e 3 do DBT (GALLAGHER et al., 1993). Como geralmente os compostos análogos ao DBT presentes nos combustíveis fósseis têm substituintes alquil ou aril nessas posições, esses compostos não poderão ser degradados por esta via. Por outro lado, o produto final da degradação parcial do DBT ainda contém o átomo de enxofre, apresentando níveis de toxicidade biológica semelhantes ao substrato inicial (GALLAGHER et al., 1993).

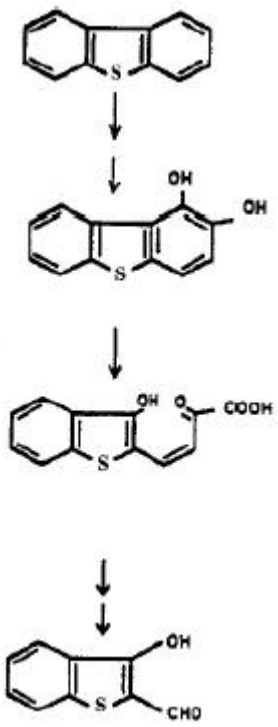

Figura 4. Via de Kodama. O átomo de enxofre não é removido havendo ruptura das ligações C-C. Fonte: ALVES, et al., 1999.

- **Via de Van Afferden**

Em 1990 Van Afferden propõe uma via metabólica diferente em *Brevibacterium* sp., na qual o DBT é convertido, em quantidades estequiométricas, a benzoato e sulfito que por sua vez é oxidado a sulfato, por oxidação abiótica (Fig. 4) (VAN AFFERDEN et al., 1993). O benzoato é, por sua vez, totalmente mineralizado a CO_2 e H_2O. Deste modo, o DBT é usado como nutriente pela bactéria no papel duplo de fonte de carbono e enxofre.

Esta via de degradação do DBT não tem grande interesse em termos de processos de biodessulfurização de combustíveis fósseis, já que a mineralização completa da estrutura carbonada implicará necessariamente uma diminuição na energia química potencial dos combustíveis. Porém, as bactérias utilizadoras desta via metabólica são potencialmente úteis na formulação de inóculos microbianos mistos para processos de bioremediação de hidrocarbonetos poliaromáticos contendo enxofre liberados no ambiente.

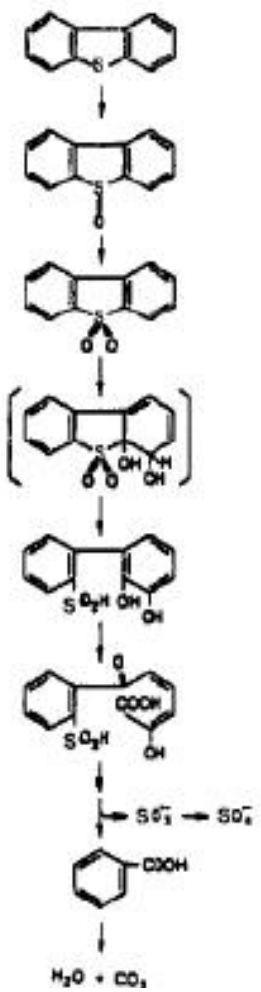

Figura 5. Via de Van Afferden. O enxofre é removido na forma de íon sulfito ocorrendo ruptura da estrutura carbonada. Fonte: ALVES et al., 1999.

- **Via do "4S"**

Uma terceira via metabólica descrita é a via sulfóxido-sulfona-sulfonato-sulfato, normalmente denominada "4S". Trata-se de uma via específica para a remoção do átomo de enxofre presente no DBT em que o grupo tiofênico sofre um ataque oxidativo progressivo. Atualmente sabe-se, a partir de estudos em *Rhodococcus* sp. IGTS8, que esta via envolve um sistema multienzimático com três atividades diferentes (Fig. 5) (GRAY et al., 1996). A primeira enzima é uma monoxigenase do DBT, que oxida o DBT a 5,5'-dióxido de DBT em dois passos; a segunda enzima é igualmente uma monoxigenase que converte o 5,5'-dióxido de DBT a 2'- hidroxibifenilo-2-sulfinato e finalmente uma lise que catalisa a quebra da ligação C-S transformando o 2'-hidroxibifenilo-2-sulfinato em dois produtos finais, 2-hidroxibifenil (HBP) e sulfato. As duas primeiras enzimas da via requerem

oxigênio molecular, NADH e FMN como cofatores. Uma terceira enzima foi recentemente descrita em *Rhodococcus* sp., uma redutase de FMN endógena responsável pelo fornecimento da flavina reduzida às monooxigenases (GRAY et al., 1996). Este novo sistema multienzimático pressupõe a existência de monoxigenases que utilizem flavina livre como substrato e a consequente existência de um complexo enzimático entre as oxigenases e a redutase de flavina. Os microrganismos que utilizam esta via para metabolizar o DBT conseguem assim que o átomo potencialmente tóxico seja retirado do composto tiofênico, sob a forma de um composto tratável (sulfato) apenas com uma ligeira perda do seu valor energético (WANG & KRAWIEC, 1994). Deste modo, as estirpes utilizadoras da via "4S" poderão constituir uma "ferramenta" biológica fundamental no tratamento em larga escala dos combustíveis fósseis, caso se consigam obter biocatalizadores de elevada estabilidade em ambiente industrial (WANG & KRAWIEC, 1994).

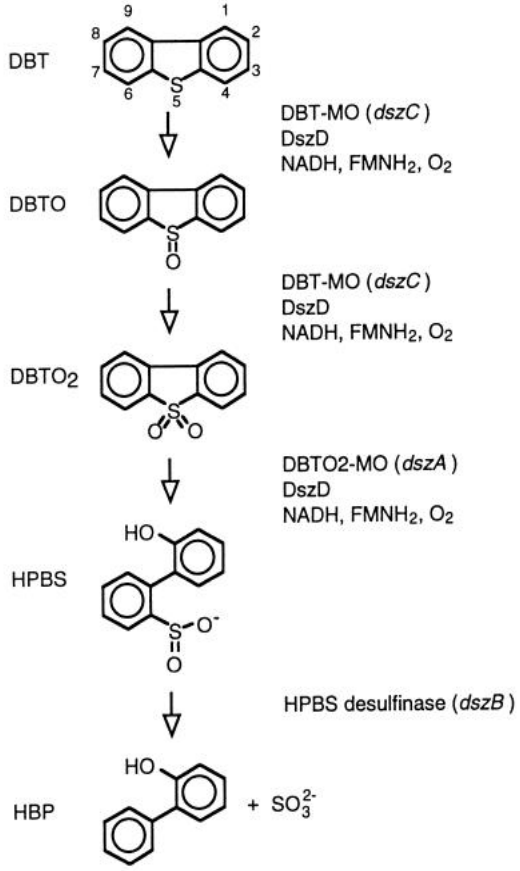

Figura 6. Via "4S". O enxofre é removido na forma de sulfito, permanecendo intacta a estrutura carbonada.

3.4. Perspectivas da Biologia Molecular e microbiologia do Petróleo

Com avanços da biologia molecular, técnicas para descrever as comunidades microbianas têm surgido e sido aplicadas nos ambientes onde microrganismos com capacidade de metabolizar hidrocarbonetos são encontrados (VAN HAMME et al., 2003).

Os potenciais metabólicos dos microrganismos têm resultado no desenvolvimento de tecnologias microbianas para o aumento da recuperação do petróleo, biorrefino e administração de efluentes petroquímicos (VAN HAMME et al., 2006).

Estudos de biologia molecular permitem conhecer as vias enzimáticas presentes no metabolismo do DBT e as linhagens que possuem alta atividade de dessulfurização (ALVES, 2007).

Gallardo et al. (1997) relatou um metabolismo mais rápido do DBT, usando uma linhagem recombinante de *Pseudomonas*; enquanto essa linhagem transformou 95% do DBT em 24 horas de incubação, apenas 18% do DBT foi transformado por *Rhodoccocus erytheropolis* IGTS8.

A via bioquímica para o metabolismo de dibenzotiofeno em linhagens de *Pseudomonas* foi descrita por Kodama et al. (1973) e resulta na degradação de um dos anéis aromáticos do DBT a 3-hidroxi-2-formil-benzotiofeno. Esta via para o metabolismo do DBT tem similaridades bioquímicas com as vias oxidativas do naftaleno, fenantreno e antraceno. Alguns genes degradativos do DBT têm se mostrado similares aos plasmídeos degradativos do naftaleno (EVANS et al., 1965; FOGHT et al., 1990; EATON & CHAPMAN, 1992).

O tratamento biológico dos compostos petroquímicos é uma área relativamente recente, e apesar dos resultados obtidos, a obtenção de um processo viável em termos industriais requer ainda uma ação conjugada de estudos de fisiologia microbiana, biologia molecular, bioquímica e engenharia química, para que a introdução de um bioprocesso se faça numa área economicamente sensível, como é a dos combustíveis fósseis. Continua a ser importante a seleção de novas estirpes microbianas com capacidade de metabolizar combustíveis fósseis, pois existem ainda muitas espécies com funções ainda desconhecidas (ALVES et al., 1999; RABUS et al., 2005).

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RESUMO

A cinética de crescimento da linhagem selvagem de *Pseudomonas fluorescens* UCP 1514 foi investigada através de experimentos em batelada contendo dibenzotiofeno (DBT), um composto recalcitrante. Neste trabalho foi investigado o desenvolvimento bacteriano em meio com alta concentração de dibenzotiofeno e seu potencial na biodegradação. O microrganismo foi crescido no meio sólido Luria Bertani com 10 mM de DBT em placas de Petri de 20ml, mantidas em posição inclinada (para que o meio atingisse 1 cm de distância da parte mais alta da borda da placa), até a solidificação do meio, para promover o gradiente de DBT. Em seguida, após a solidificação do gradiente de DBT foi adicionada uma suspensão de 10^6 UFC/ml de *Pseudomonas fluorescens*, em 9,9 ml do meio, sendo as placas mantidas em posição horizontal. As placas foram mantidas em estufa a 37°C durante 24 horas. Após o período de incubação foram observadas a presença de colônias viáveis e resitentes. Após a seleção, as colônias foram mantidas no meio líquido contendo 2 mM de DBT. Para a realização da cinética de crescimento, o microrganismo foi crescido em meio Luria Bertani (LB), durante 48 horas. Foram acompanhados o pH e a viabilidade celular através da técnica de "Pour plate", usando o meio ágar nutriente. Em seguida foi avaliada a velocidade máxima de crescimento e tempo de geração das linhagens selvagem e mutante fisiológico. Os resultados demonstraram que o início da fase exponencial foi observado entre 24 e 72 horas de cultivo. A velocidade específica de crescimento foi de 0,15 h^{-1} para o controle e 0,39 h^{-1} para o meio com DBT, com tempo de geração de 4,62 e 1,77h, para o controle e o meio com DBT, respectivamente. O pH não sofreu grandes modificações. *Pseudomonas fluorescens* UCP(1514) apresentou um perfil de crescimento semelhante no controle e no meio com DBT. No controle, o crescimento foi observado até as 72 horas de cultivo com 0,23 g/l, depois deste período, começou a fase de declínio do microrganismo, com 0,17 g/l a 144 horas. No entanto, nos experimentos com dibenzotiofeno, o oposto ocorreu; depois do tempo de adaptação o microrganismo continua a aumentar e manter sua população, apresentando 0,35 g/l a 144 horas. O microrganismo foi capaz de metabolizar o dibenzotiofeno. O DNA das linhagens bacterianas selvagem e mutante fisiológico foi amplificado pela técnica de reação em cadeia da polimerase (PCR), com o oligonucleotídio BOX. A amplificação do DNA genômico permitiu realizar a fenotipagem molecular

proporcionando uma discriminação acurada entre as colônias selvagens e as mutantes fisiológicas, demonstrando grande diversidade genética.

Biochemistry strategies of *Pseudomonas fluorescens* on metabolism and growth in medium with dibenzothiophene

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Abstract

The growth kinetics of a bacterium of the strain *Pseudomonas fluorescens* UCP 1514, which is indigenous in soil, has been investigated in batch cultures containing dibenzothiophene. In this paper, we discuss the potential of the bacterium, when grown in medium with high concentration of dibenzothiophene and its potential for biodegradation. The microrganism was grown in medium Luria Bertani – agar medium with 10mM of DBT during 24hours, to select resistant colonies. After selection of the colonies, the microorganism was maintained in liquid medium with 2 mM of DBT dissolved in dimethylformamide. The kinetics of growth was evaluated through viability and pH. The results showed that the microorganism was able to metabolize DBT. We also identified the physiological mutant colonies that grew on DBT by DNA amplification using the PCR (polymerase chain reaction) technique and the specific primer BOX.

Keywords: Dibenzothiophene, Biodegradation, Kinetics growth, *Pseudomonas fluorescens,* DNA profile, physiological mutant strain

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) comprise a large and heterogeneous group of organic contaminants that are formed and emitted as a result of the incomplete combustion of organic material. Anthropogenic sources such as road traffic, combustion of fossil fuels and oil spills are the most relevant sources, but there are also natural ones such as forest

fires. PAHs consist of fused benzene rings and/or pentacyclic molecules in linear, angular or clustered arrangements. PAHs have been thoroughly studied due to their toxicity and environmental persistence. Such studies are often limited to 16 PAHs, designated as priority pollutants by the United States Environmental Protection Agency (US-EPA).

The bioremediation of soil contaminated with PAHs is often limited by the PAH bioavailability of PAH due to their low aqueous solubility and high soil–water distribution ratios, which attest to their ready microbial utilization and promote their accumulation in solids phases of the terrestrial environment [1].

Organosulfur compounds, such as dibenzothiophene (DBT) and benzothiophene (BT), in fossil fuels are released as sulfur oxyacids by combustion and cause environmental problems such as acid rain. Furthermore, such oil comtamination may impact on the organisms that live in contaminated ecosystems because some of these compounds, are reported as having mutagenicity and carcinogenicity [2].

Therefore, research regarding the degradation or desulphurization of such thiophenic compounds would be necessary for environmental aspects [3]. Considerable development, in recent years, has occurred in the application of Molecular Biology Techniques, especially in PCR analysis (Polymerase Chain Reaction), which allows the amplification of given sequences of DNA molecule. In research on genetic diversity, PCR analysis has often been used with specifics primers, such as BOX, ERIC and REP, and the profiles obtained have resulted in products after reaction which allow the strains to be differentiated [4 -11].

Genetic variation within rhizobia was assessed using BOX-PCR and was shown to be a useful method for differentiating closely related rhizobia. This method was used to assess whether the presence of heavy metals in soil led to noticeable changes in rhizobia diversity.

BOX-PCR analysis was preferred to other genetic approaches such as (16S-DNA analysis) due to its ability to detect DNA mutations, insertions and deletions [12].

In this investigation we assess the ability of the bacterium *Pseudomonas fluorescens* to grow in the presence of dibenzothiophene and its influence on microbial metabolism. We studied the DNA amplification using the PCR (Polymerase Chain Reaction) technique and the specific primer, BOX. Since dibenzothiophene (DBT) derivatives are the most frequently encountered in organic sulfur compounds, DBT is generally employed as a model molecule in microbiological studies of heavy oil desulfurization.

2. Materials and methods

Microorganism and culture medium

The experiments were carried out using pure cultures of *Pseudomonas fluorescens* (UCP 1514) deposited in the Nucleus of Research in Environmental Sciences – NPCIAMB, of the Catholic University of Pernambuco. The bacterial culture was kept in solid medium agar nutrient consisting of meat extract (5.0g), peptone (10.0g), NaCl (5.0g), agar (15.0) per litre of distilled water, at 4°C as a stock of bacterial innoculum.

Selection of physiological mutant colonies to DBT

The strain of *Pseudomonas fluorescens* was submitted with a gradient of DBT, using the technique described by Szybalski and Bryson [13]. The culture medium used for performing the technique was Luria Bertani (LB), containing tryptone (10.0g), yeast extract (5.0g), NaCl (10.0g) and glucose (3.0g), agar (17.0g) per litre, with pH 7.0, as per Konish et al. [14]. The solution of DBT (Sigma-Aldrich®) it was prepared in dimethylformamide (DMF) in a concentration to 1 M filtered and sterilized in a Millipore® filter.

To the culture medium was added 1ml of DBT in the concentration of 10mM in Petri plates kept in sloping position (so that the half reached 1 cm away from the part of

most discharge on the border of the plate), until the DBT gradient solidified. Then, after solidification of gradient DBT, a suspension of 10^6 UFC/ml of *Pseudomonas fluorescens* was added in 9.9 ml of medium, being the plates maintained in horizontal position.

The plates were kept at 37°C for 24 hours. After incubation time, the colonies grown in higher concentration of DBT were observed. All experiments were followed in triplicate and with control. The, the physiological mutant colonies were transferred for tubes containing culture medium LB with DBT at 10mM.

Batch experiments

The culture was grown in LB liquid medium as described earlier. The following DBT concentration and solvent was employed as stock solution: 1M in dimethylfomamide (DMF) [15].

The solution was sterilized in a Millipore® filter. The Erlenmeyer flasks were autoclaved at 120°C for 30 minutes. Batch kinetic experiments were conducted in 250 ml Erlenmeyer flasks by adding 2 mM DBT solution to 100 ml liquid culture medium. Another culture was prepared as innoculum just before batch experiment and, while the bacteria were in the exponential phase, a volume of 5ml consisting of 10^8 cells/ml was transferred to 100 ml of liquid medium.

All experiments were followed in triplicate by control trials without innoculum, performed on a rotary shaker operating at 150 rpm at a constant temperature of 37°C for 144 hours. Samples were taken every 24 hours. Bacterial growth was monitored through cell viability using the pour plate technique and the pH by using a pHmeter (Orion 310). The specific growth speed (μ_{esp}) and generation time (T_G) were determined as per Pirt [16]. For the specific growth rate, the following equation was used:

$\mu_{esp} = (\ln X - \ln X_0 / T - T_0)$,

Where:

X= Final biomass

X_0= Initial biomass

T= Final time

T_0= Initial time

The generation time was determined by:

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

After culture time, the fermentations were centrifuged at 5,000 *g* for 15 minutes at 5°C to separate the biomass and metabolic liquid. After separation, the metabolic liquid was utilized to determinate the consumption of glucose and the quantification of protein.

Analytical methods

The glucose consumption was determined by the colorimetric method (LABTEST® Diagnotisc-Brazil) at 505 nm and the total protein was determined as per the Bradford [17] method, using Bovine Serum Albumin (BSA) as standard. The reaction system was performed by 1ml of staining solution (Bradford – Coomassie Brilliant Blue G-250 100mg/l, ethanol 95% and HPO_4 85%) and 100µl of sample. The mixer was homogenized and the absorbance at 595nm was verified. A calibration curve with (BSA) was drawn as a reference.

Molecular biology studies

Genomic DNA extraction

As to DNA extraction, the microorganism was grown in Luria Bertani-liquid medium at 37°C with 150 rpm at an early exponential state. After fermentation, the cells were put on ice during 15 minutes, then had been centrifuged at 13,000 *g*, at 4°C for 10 minutes.

The supernatant was removed and the cells, washed by adding 50µl of isotonic solution and performing another centrifugation (13,000 *g* , 4°C for 10 minutes), were resuspended in 1/50 the volume of isotonic solution. The experiment was performed by adding cell lysis solution, equal to the volume of the resuspended solution, mixed by reversal and put on ice for 5 minutes.

The sample was extracted with chloroform isoamilic alcohol in 24:1(v/v), mixed by reversal, centrifuged as described earlier and the organic phase was omitted. The DNA was exracted in the aqueous phase with 1:3 the volume of ammonium acetate (7.5M), mixed by reversal, adding 2,5 times the volume of ethanol, mixed again by reversal and kept it for 15 minutes at room temperature; it was centrifuged for 15 minutes at 12,000 rpm and the supernatant removed by adding 1ml of alcohol 70%, centrifuged at 13,000*g* , 4°C for 5 minutes, and thereafter dried at room temperature. The DNA was dissolved in TBE buffer and quantified in agarose gel to 1%, stained with SyBr Gold 1X (Invitrogen®).

Electrophoresis was performed in a horizontal system using TBE buffer 0.5 X for 50 minutes at 80 Volts. The gel was visualized in a UV chamber.

DNA amplification

The DNA was amplified by PCR (Polymerase Chain Reaction) with primer BOX(5'-ACGGCAAGGCGACGTCGACG-3'), which conserved amplifying conserved and repetitive regions of cromossomic DNA [18]. The amplification reaction was performed with the following volumes: milli-Q sterile water, 16.36 µl; dNTPs, 0.4 µl (stock solution with 20mM each base); buffer 10X, 2.5 µl; $MgCl_2$, 1.25 µl (50mM); oligonucleotide, 1.0 µl (50pmol μl^{-1}); DNA, 1.0 µl (50mg); Taq, 50 µl (5U/ µl).

The amplification was performed using the following cycles: (i) one cycle of initial denaturation 7 min at 95°C; (ii)35x [denaturation 1min at 94°C, hybridization 1min at

53°C, extension 8min at 65°C]; (iii) one cycle of final extension 16 min at 65°C, maintained at 4°C.

The samples were then submitted to gel eletrophoresis in agarose gel, at 1.5% (20x25cm), at 100V in TBE buffer 1X (10.8 g Tris-base 5.5g boric acid , 4ml of EDTA 0.5M pH 8.0, per liter of solution) that, after six hours, stained with ethide bromide and visualized in a UV chamber [19]. The bands were analyzed by Biometra Program 65.

Electrophoresis of proteins SDS-PAGE

The analysis were performed in line with Laemmli [20]. The samples were diluted in buffer (Tris-HCl 12mmol/l pH6.8, glycerol 5%, SDS (Sodium dodecyl sulphate) 0.4%, 2-mercaptoethanol 2mmol/l and bromophenol blue 0.02%) and denatured at 90°C for 5 minutes. Aliquots were applied in polyacrylamide gel containing SDS.

The concentration of polyacrylamide on separator and ordered gel were12% and 14%, respectively. The ratio of acrylamide to bisacrylamide was 30:8 (w/w). The separator gel contained 2.5ml of Tris-HCl 1.5mol/l pH 8.8, 100ml of SDS 10% (m/v), 50µl of ammonium persulphate 10%(m/v) and 5µl of TEMED. The Electrophoresis was performed at 150V for 2 hours in Tris-HCl buffer, 25mmol/l pH8.3, glycine 192mmol/l and SDS 0.1%, using Höefer Gel Systems.

The gels were stained with Coomassie Brilliant Blue solution, 250 R (Coomassie Blue 0.15%, methanol 53% and acetic acid 7% in bi-distilled water. The gels were immersed in a dryer solution (methanol 50% and glycerol 1% in bi-distilled water) for 2 hours.

Results and discussion

The microorganism *Pseudomonas fluorescens* was isolated from soil contaminated with petroleum waste and it is capable of growing at a high concentration of DBT. These

strains (wild and mutant) can be use DBT as the only source of sulfur. And the utilization of sulfur from DBT is not dependent on the presence of glucose in the culture medium. The results showed greatest growth of *P. fluorescens* at DBT concentration of 2mM, but the microorganism is unable to grow when the concentration is higher than 5mM, in liquid medium, possibly as due to toxicity.

Most hydrocarbon, the alkanes and aromatic and polyaromatic compounds in petroleum are sources of energy and carbon for producing cellular materials. Microorganisms may also satisfy their nitrogen and sulfur requirements by metabolizing compounds such as carbazole [21] and dibenzothiophene [22].

In many cases, hydrocarbon-oxidizing enzymes such as toluene monooxygenase are nonspecific and may oxidize non-growth substrates as a byproduct of normal metabolism, a phenomenon termed cometabolism. Since the molecular structures found in hydrocarbon mixtures are so diverse, it is not surprising that microorganisms have a vast number of enzymes for petroleum hydrocarbon metabolism [23].

Kinetic study and effect of DBT on the growth of Pseudomonas fluorescens

The colonies visualized after growth period with DBT on the Petri plates did not show morphological alteration, maintaining the structures and sizes characteristic. During the fermentation, *Pseudomonas fluorescens* UCP 1514 grew as well both in the control as in DBT medium (Figure 1). The beginning of the exponential phase was observed at 6 hours of culture.

The specific velocity of growth on control was 0.25 h^{-1} and in experiments with DBT was 0.22 h^{-1}, with a generation time of 2.77 and 3.15 hours, for the control and DBT culture, respectively (Table 1).

The pH was not modified significantly during the cultivation. On control, the growth was observed until 48 hours of culture. After this period, the decline phase of microorganisms began.

However, in experiments with dibenzothiophene, the opposite occurred; after adaptation time, the microorganism continued to increase its population, as well as maintaining it, probably due to the utilization of hydrocarbon and its metabolites as the main energy source [24].

The protein concentration was analyzed on the samples of metabolic liquid extracted each 2 hours until 12 hours of culture and thereafter at periods of 24 hours until 144 hours, at the of fermentation. Kumaran and Paruchuri [25] have studied *P. fluorescens* kinetics for biodegradation of phenol in batch experiments at 30 °C.

They used both Monod's and Andrews's model for the specific growth rate and concluded that μmax calculated by Andrews's model should be considered as the true value. The value of μ_{max} was 0.618 h^{-1}, whereas K_s and K_i were 71.4 and 241 mg/l, respectively. A review of the literature showed considerable variance in the values of the kinetic parameters.

Grady et al. [26] mentioned that the main reasons for this variance are culture history, parameter identifiability and the nature of the assay procedure employed to measure the parameters. Furthermore, Sommer et al. [27] found that reproducibility of biological experiments is rather limited, because it is difficult to assure fixed environmental conditions and to prevent changes in the physiological state of the microorganisms.

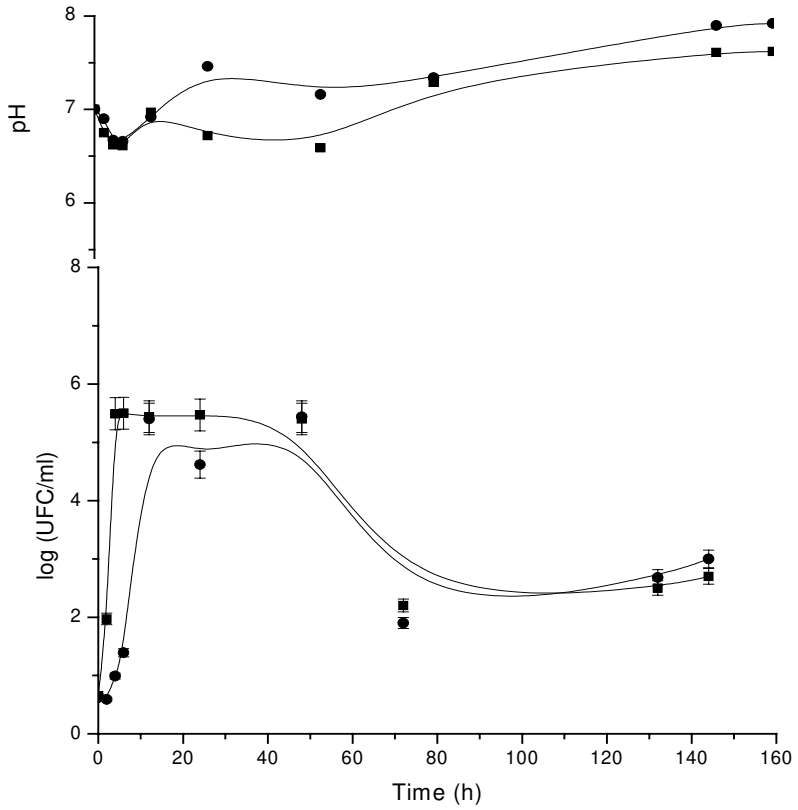

Figure 1. Kinetic growth and pH of *Pseudomonas fluorescens* in Luria Bertani medium. (■) Control; (●) 2mM DBT.

Table1. Values of specific velocity of growth μ_{esp} and generation time T_G of kinetic growth of *Pseudomonas fluorescens* in Luria Bertani Medium during 144 of culture.

Experiments	μ_{esp}	T_G
Control	0,25 h^{-1}	2,77 h
Medium with 2mM DBT	0,22 h^{-1}	3,15 h

Therefore, the differences between the kinetic parameter values estimated here and the ones reported in the literature can be considered as reasonable. The Figure 2 shows that the extracellular proteins produced increased after 24 hours of culture.

Then the microorganisms produce an expressive proportion of enzymes to bring the adaptation to compound, as well as about to degrade it with the cleavage carbon-carbon bonds, and then reducing the compounds less complex [28].

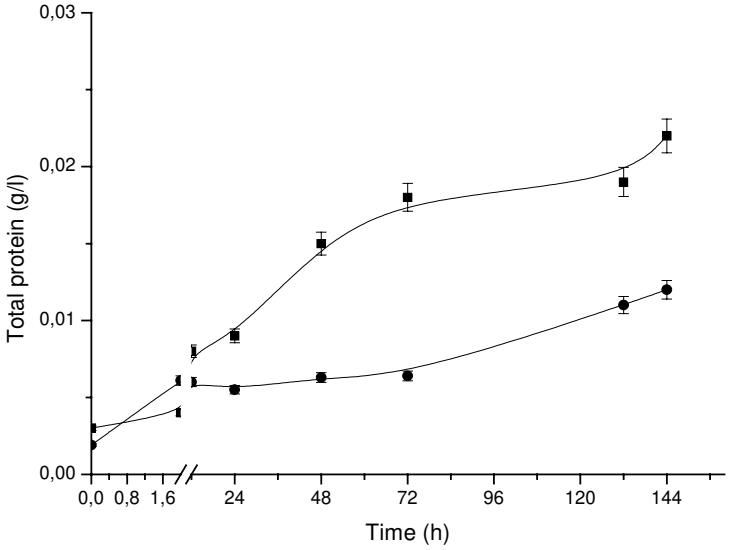

Figure 2. Total protein during metabolism of *Pseudomonas fluorescens* in Luria Bertani medium. (■) Control; (●) 2mM DBT.

The same behavior was observed in *Pseudomonas fluorescens*, at first on adaptation period to the compound that resulted in a production of extracellular proteins. In presence of recalcitrant compounds, in the beginning of the growth, the microorganisms seek to adapt and after utilizing the compounds as carbon source.

The glucose consumption was expressive at begin of the fermentation as shown in Figure 3, due to the intensive activity of microorganism for remain viable in presence of dibenzothiophene and then, degrade it. The glucose was consumed practically at first hours of fermentation, due to the glucose to be an easy source of energy and carbon, confirming that in this period the dibenzothiophene was used by *Pseudomonas fluorescens*, probably, as energy source and even sulfur, to biosynthesis of vitamins and aminoacids [29].

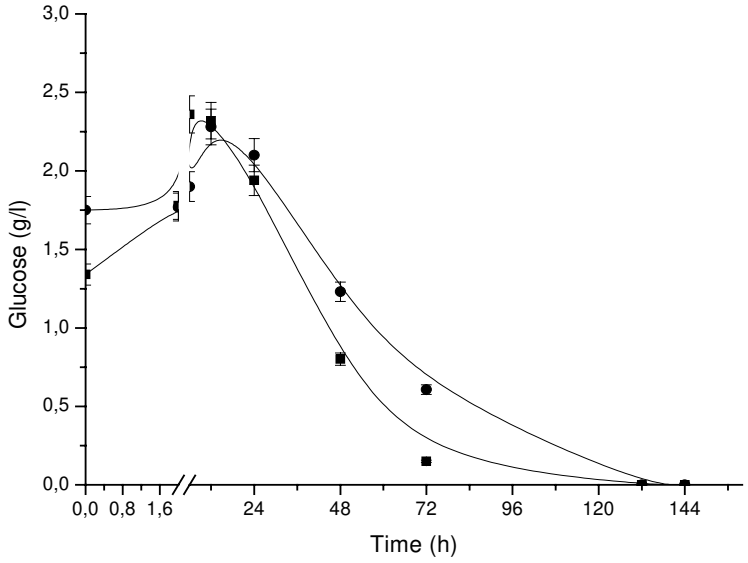

Figure 3. Glucose consumption during metabolism of *Pseudomonas fluorescens* in Luria Bertani medium. (■) Control; (●) 2mM DBT.

Gallardo et al, [30] described a metabolism of DBT using a recombinant *Pseudomonas* strain that transformed 95% of the DBT at 24 hours of incubation, while *Rhodococcus erythrhopolis* strain IGTS8 transformed only 18% of the DBT.

So, this bacterium has potential to be used in biodesulfurization studies of fossil fuels since it not decrease the carbon content of desulfurized fuel. Sgountzos et al., [31] studied the growth of *P.fluorescens* in batch systems and compared with sand beds during biodegradation of phenol and the studies demonstrated that quantitatively, the batch system, as the present study, is better.

The results presented that the bacterium grew faster and consumed phenol at higher rates in batch experiments than in columns systems. This was because in a batch system homogeneous conditions can be obtained so that the role of mass transfer is weaker than that in packed beds. Also, in the batch systems, oxygen was in excess in contrary to the packed bed experiments, where oxygen availability was limited.

The packed bed experiments also showed that microbial growth processes in porous media were difficult to be reproduced quantitatively. However, if there is good control of the key parameters and conditions, the experiments reveal the dynamic nature of these processes and help to understand and characterize the growth kinetics of microorganisms.

Molecular biology studies

The strain wild of *Pseudomonas fluorescens* that grown on gradient of DBT was analyzed comparing with control. Molecular biology studies were realized to identify the modifications on physiology and metabolism of microorganism.

Were realized the DNA amplification by PCR and electrophoresis of proteins with SDS-PAGE on the wild strain of *Pseudomonas fluorescens*, the strain maintained with 10 mM of DBT and this same sample added in liquid medium Luria Bertani also with DBT at 10mM.

The comparison with the standard of amplification of the genomic DNA fragments generated by repetitive elements amplified by primer BOX is shown in Figure 4.

The reaction of amplification of DNA genomic region of *Pseudomonas fluorescens* way PCR, using primer BOX demonstrated a significant number of bands. In gram-negative bacteria, the nucleotides ERIC and BOX were also used successfully on identification of genus *Xanthomonas* and *Pseudomonas* [32] and genetic diversity between the isolated of *Ralstonia solanacearum* [33].

Figure 4 shows that the bands varied from 400 to 2000kb. The wild strain presented products of amplification about of 650 to 2000kb, while the strain treated with DBT shown products of amplification between 400 to 2000kb.

The band of molecular mass of 500kb is very expressive on samples 1 and 2, treated with DBT, but not appeared on sample 1, wild strain. We may to note that the band of 100pb of the marker, presents three different forms on the bands.

According to Denome et al [34], a 9.8kb DNA fragment cloned from a pseudomonad soil isolate (C18) was found to encode proteins capable of metabolizing DBT, naphthalene and phenanthrene.

This DNA was sequenced and found to contain 10 open reading frames (ORF_S) greater than 100 aminoacids in length. They designated these $ORF_{S\ the}$ DOX pathway (DBT oxidation), since they were cloned by their ability to degrade DBT. Sequence analysis revealed that DOX encodes proteins similar to those that degrade other aromatic compounds, including naphthalene, benzene, biphenyl and toluene.

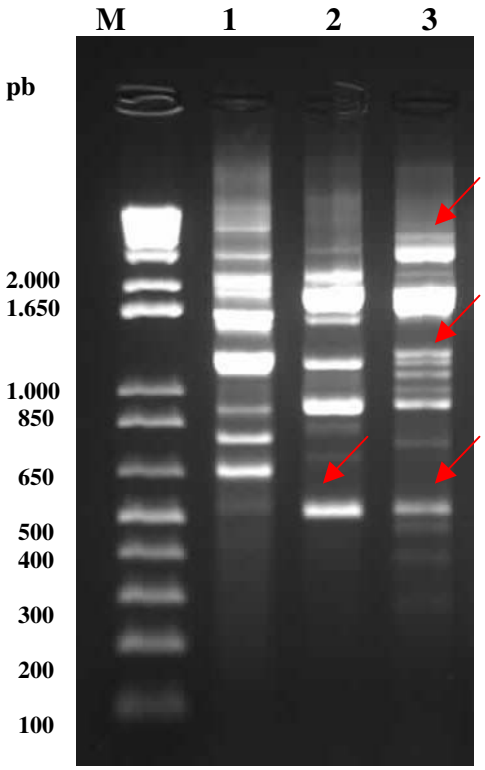

Figure 4. DNA profile 1Kb Plus DNA Ladder PCR (primer BOX). M. Marker; 1.Strain *Pseudomonas fluorescens* without DBT; 2. Strain with 10mM DBT; 3. Strain with 10mM DBT with liquid medium.

The activity of total proteins in denaturing gel (SDS-PAGE) of *Pseudomonas fluorescens* grown in Luria Bertani medium at 30°C identified the proteins. The profile obtained of samples showed different bands with proteins with molecular mass differentiates.

The molecular weight of sample 1, 2 and 3 in Figure 5 were determined to be 116; 97.4; 97.4 kDa, respectively. The results showed that *Pseudomonas fluorescens* presented a physiological mutation in its genomic profile, occurring a reorganization of the genes.

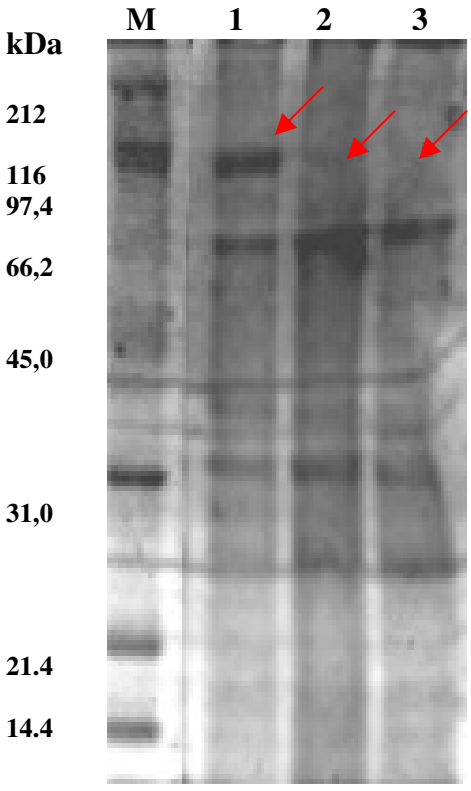

Figure 5. Protein profile. SDS-PAGE. M. Marker; 1.Strain *Pseudomonas fluorescens* without DBT; 2. Strain with 10mM DBT; 3. Strain with 10mM DBT with liquid medium.

Oshiro et al., [35] studied the dibenzothiophene desulfurizing enzymes and they purified three enzymes involved in the microbial DBT desulfurization from the thermophilic bacterium *B. subtilis* WU-S2B.

It has been reported that the thermophilic desulfurizing strain *Mycobacterium phlei* GTIS10, grew at 50°C and the genes involved in the desulfurization were identical to those of the mesophilic desulfurizing bacterium, *R. erythropolis* IGTS8 [36].

This thermotolerance was explained by the property of the bacterial host strain; namely, it was claimed that the GTIS10 strain exhibited a desulfurizing activity at higher temperature because it had higher abilities of supplying cofactors for the enzyme reactions and of transporting substrates and products compared with the IGTS8 strain.

However, they confirmed that the desulfurizing enzymes from *B. subtilis* WU-S2B had higher activities and stabilities at high temperature than those from the mesophilic desulfurizing *Rhodococcus* strain.

It has been reported that the activity of a desulfurization enzyme was improved using the DNA shuffling method [37], and that the amino acid residue involved in the substrate recognition of genes involved in the desulfurization of DBT was identified by a site-directed mutagenesis study [38].

These results suggest that, in implementing this genetic screen, we have to identify heretofore unknown genes and optimizing the enzymatic activity would be necessary by the genes and enzymes analysis responsible for desulfurization. Structural analyses of enzymes for DBT desulfurization are in progress, and such genetic engineering techniques will enhance and improve the desulfurizing enzymes, making them suitable for use in novel biodesulfurization processes.

Acknowledgements

This work was supported by the Brazilian National Council for Technological and Scientific Development (CNPq) and FINEP (Financial Agency of Studies and Projects).

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CAPÍTULO III

DESSULFURIZAÇÃO DO DIBENZOTIOFENO POR *PSEUDOMONAS FLUORESCENS* PRODUZINDO BIFENIL

Manuscrito submetido para publicação no:
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RESUMO

O potencial de *Pseudomonas fluorescens* na dessulfurização do dibenzotiofeno (DBT), um componente recalcitrante dos combustíveis fósseis foi estudado, visando uma possível aplicação na redução de enxofre contida no óleo diesel, em conformidade com as regulamentações ambientais. Os experimentos foram realizados utilizando uma cultura pura de *Pseudomonas fluorescens* (UCP 1514A), pertencente ao banco de culturas do Núcleo de Pesquisas em Ciências Ambientais - NPCIAMB, mantidas 4º C na Universidade Católica de Pernambuco. A cultura foi mantida em meio sólido agar-nutriente, consistindo de extrato de carne (5,0g), peptona (10,0g), NaCl (5,0g), agar (15,0g), DBT (2mM), por litro de água destilada. O microrganismo foi crescido em meio Luria Bertani – LB contendo por litro triptona (10,0g), extrato de levedura (5,0g) e cloreto de sódio (10,0g)com o pH 7.0. A solução do DBT foi preparada em dimetilformamida numa concentração a 1M, filtrada e esterilizada em filtro Milipore. Os frascos de Erlenmeyer foram autoclavados a 120ºC por 30 minutos. Os experimentos em batelada foram conduzidos adicionando 2mM da solução de DBT em 100ml do meio de cultura líquido LB. O pré-inóculo foi crescido durante 12 horas e inoculado 5ml, consistindo de 10^8 células/ml, no meio líquido. Todos os experimentos foram feitos em triplicata, seguidos de um controle, em Shaker a 150 rpm, temperatura constante de 37ºC, durante 144 horas. Alíquotas foram retiradas a cada 24 horas para o monitoramento do crescimento através de biomassa e pH. Para a realização da cinética de crescimento foram acompanhados a viabilidade celular e pH, sendo determinados a velocidade específica de crescimento e o tempo de geração. Para avaliação da biodegradação do DBT pelo microrganismo, a cultura de *Pseudomonas fluorescens* foi crescida em meio líquido, a 37°C sob agitação em shaker orbital (150 rpm) por diferentes períodos de tempo. Após o período de crescimento, as amostras foram centrifugadas A 5.000 *g* por 15 minutos a 5ºC e o sobrenadante foi extraído com acetato de etila. A camada orgânica foi removida e a camada aquosa foi acidificada a um pH 2 com 5 N HCl e novamente extraída com acetato de etila. Ambos os extratos foram reunidos e evaporados em um rotaevaporador e ressuspendidos em 500µl de acetato de etila. A quantificação do DBT utilizado pela bactéria foi evidenciada por cromatografia líquida de alta performance (HPLC). Os metabólitos produzidos pela dessulfurização foram identificados por cromatografia gasosa acoplada a um espectrômetro de massa (GC-MS), observando-se a

presença de um produto livre de enxofre, bifenil. Os resultados obtidos apresentaram um decréscimo de 73% na concentração inicial de dibenzotiofeno, indicando que *Pseudomonas fluorescens* remove seletivamente o enxofre do DBT para formar bifenil. Neste sentido, perspectivas biotecnológicas indicam que esta linhagem bacteriana apresenta um potencial promissor para minimizar a concentração de enxofre contido nos derivados de petróleo, particularmente, após o processo de hidrodessulfurização, o qual contribuiria com o cumprimento das regulamentações dos padrões ambientais dos níveis de enxofre contido nos combustíveis fósseis.

Desulfurization of Dibenzothiophene by *Pseudomonas fluorescens* (1514A) leading to the production of biphenyl

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Abstract

The potential of *Pseudomonas fluorescens* (1514A) on the desulfurization of Dibenzothiphene, a recalcitrant component of fossil fuels was studied, in order to use it for reducing the sulfur content of diesel oil in compliance with environmental regulations. The biodegradation of dibenzothiophene by the bacteria was determined by high performance liquid chromatography. Metabolites produced by dibenzothiophene desulfurization were identified by gas chromatography – mass spectrometry, and a sulfur-free product, biphenyl, was detected from degradation process. The results showed a decrease of 73 % in dibenzothiophene content, indicating that *P. fluorescens* selectively removes sulfur from that compound to form biphenyl, indicating that this bacterium shows promising potential for decreasing the sulfur content of diesel oil. The process of microbial desulfurization described herein can be used, thus significantly reducing the sulfur content of oil, particularly, after the process of hydrodesulfurization which would help in understanding and meeting the regulatory standards for sulfur level in fossil fuels.

Subject Category: Microbial engineering

Keywords: Biodegradation/ Biodesulfurization/ Dibenzothiophene/ Diesel/ *Pseudomonas fluorescens*

Introduction

Direct combustion of fossil fuels leads to sulfur oxide emissions that contribute to acid rain and air pollution (Li, *et al*., 2007). Dibenzothiophene (DBT) and its derivates are the main organic sulfur compounds existing in petroleum.

As these heterocyclic compounds are relatively stable, the traditional processes, such as acid alkali treatment, are inefficient to remove 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 appeared to be expensive both in its capital investment and in the operating costs (Qu, et al, 2006).

Environmental regulations concerning the sulfur content of motor fuels are becoming increasingly stringent in order to reduce SO_X emissions. To comply with such regulations, Hydrodesulfurization (HDS) has been used to reduce the sulfur composition (Borgne, et al, 2003).

One strategy for reducing the sulfur content is to expose these substrates to micro-organisms that can specifically break carbon-sulfur bonds, thereby releasing the sulfur in a water-soluble, inorganic form. This process of microbial desulfurization or bio-desulfurization (BDS) is expected to overcome the technical and economic problems associated with HDS as it has the potential benefits of lower capital investments and operating costs and will produce less harmful greenhouse gases (Monticello, 2000).

As the sulfur in a thiophenic component accounts for a substantial proportion of the sulfur which remains in the fuel after HDS, and dibenzothiophene (DBT) is a typical recalcitrant sulfur component of fossil fuels, desulfurization of DBT has been a model reaction in the treatment of fossil fuels (Gallagher et al, 1993; Oldfield et al, 1997).

Research on biodesulfurization using DBT has resulted in the elucidation of two different biochemical pathways, named Kodama (Kodama et al, 1973) and 4S (Izumi et al, 1994; Kertesz et al, 2001; Oldfield et al, 1997; Omori et al, 1992; Purdy et al, 1993; Wang et al, 1994).

The Kodama pathway is considered unsuitable because water-soluble sulfur compounds are produced, which are then unavailable for burning and are therefore forfeited from the caloric value of the fuel. Through the 4S pathway, DBT is transformed to 2-Hydroxybiphenyl (2-HBP) and sulfite.

In the pathway the carbon skeleton of DBT is released intact as 2-HBP; therefore, the fuel value is not lost (Mohebali et al, 2007). There are several studies of bacterial species that can degrade DBT, including *Pseudomonas* sp. (Monticello et al, 1985; Bressler and Fedorak, 2001a), *Sphingomonas* sp. (Nadalig et al, 2002), *Burkoholderia* sp. (Gregorio et al, 2004), *Rhodococcus*sp. (Oldfield et al, 1997) and *Mycobacterium* sp. (Okada et al, 2002; Li et al, 2003).

We report the desulfurization of DBT by *Pseudomonas fluorescens*. This microorganism was found to degrade DBT via the sulfur-selective pathway and hence was applied for the desulfurization of diesel oil. The bacteria could appreciably decrease the sulfur on DBT indicating its potential for use as biocalysts in desulfurizing of fossil fuels.

Materials and methods

Microorganism and culture conditions

The experiments were carried out using pure culture of a physiologic mutant of bacterium *Pseudomonas fluorescens* (UCP 1514A) 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) per litre of distilled water, to which was added 2 mM DBT concentration at 4°C as a stock of bacterial innoculum.

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) per litre as supplement, as per Konish *et al.* (2000). The following DBT concentration and solvent was employed as stock solution: 1 M in dimethylfomamide (DMF) (Setti et al, 1994). The solution was sterilized in a Millipore® filter.

The Erlenmeyer flasks were autoclaved at 120°C for 30 minutes. 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 innoculum just before batch experiment and, while the bacteria were in the exponential phase, a volume of 5 mL consisting of 10^8 cells/mL was transferred to 100 mL of liquid medium. All experiments were made in triplicate followed by control trials without innoculum, performed on a rotary shaker operating at 150 rpm at a constant temperature of 37°C for 144 hours. Aliquots were obtained every 24 hours.

Bacterial growth was monitored through biomass and pH using pHmeter (Orion 310). The specific growth speed (μ_{esp}) and generation time (T_G) were determined according to Pirt (1975). For the specific growth rate, the equation used was:

$\mu_{esp} = \left(\ln_X - \ln_{X_0} / T - T_0 \right)$

Where,

$X =$ Final biomass

$X_0 =$ Initial biomass

$T =$ Final time

$T_0 =$ Initial time

The generation time was determined by:

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

After culture, the samples were centrifuged at 5,000 *g* for 15 minutes at 5°C to separate the biomass and metabolic liquid. After separation the biomass was liofilized to determine the dry weight of biomass per unit volume.

HPLC and GC-MS analysis

In order to determine the intermediates of the pathway of DBT bio-desulfurization by microorganisms, the bacterial culture was grown at 37°C on a rotary shaker at 150 rpm for different periods, and were then centrifuged at 5,000 *g* for 15 minutes 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 re-suspended in 1 mL of ethyl acetate.

DBT quantification was carried out 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 HPLC - Varian UV-VIS detector, model 320. Separation was carried out with a C_{18} RP column (4.6 x 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 (Abbad-Andaloussi *et al*., 2003). Gas chromatography (GC) analysis was performed on a VARIAN STAR 3600CX with a DB-1 (100% dimethylpolysiloxane) fused capillary column (30 m x 0.25 mm); column temperatures were programmed from 50°C for 5 min, raised 10°C min^{-1} , 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 mL 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^{-1} 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 Spectral Database for Organic Compounds SDBS library.

Results

Effects of DBT on the growth of Pseudomonas fluorescens

Pseudomonas fluorescens UCP 1514A grew as well as in the control as in DBT medium (Figure 1).

The beginning of the exponential phase was observed between 24 and 72 hours of culture. The specific velocity of growth on control was 0.65 h^{-1} and in experiments with DBT was 0,2h^{-1}, with a generation time of 0.027 and 0.006 hours, to control and DBT culture, respectively (Table 1).

The pH did not decrease during the cultivation. On control, the growth was observed until 72 hours of culture with 0.23g dry cell L^{-1}, after this period, the decline phase of microorganisms began, with 0.17 g dry cell L^{-1} at 144 hours. However, in experiments with dibenzothiophene, the opposite occurred; after adaptation time, the microorganism continued to increase its population, presenting 0.35g dry cell L^{-1} at 144 hours as well as maintaining it, probably due to the utilization of hydrocarbon and its metabolites as the main energy source (Kropp et al, 1997).

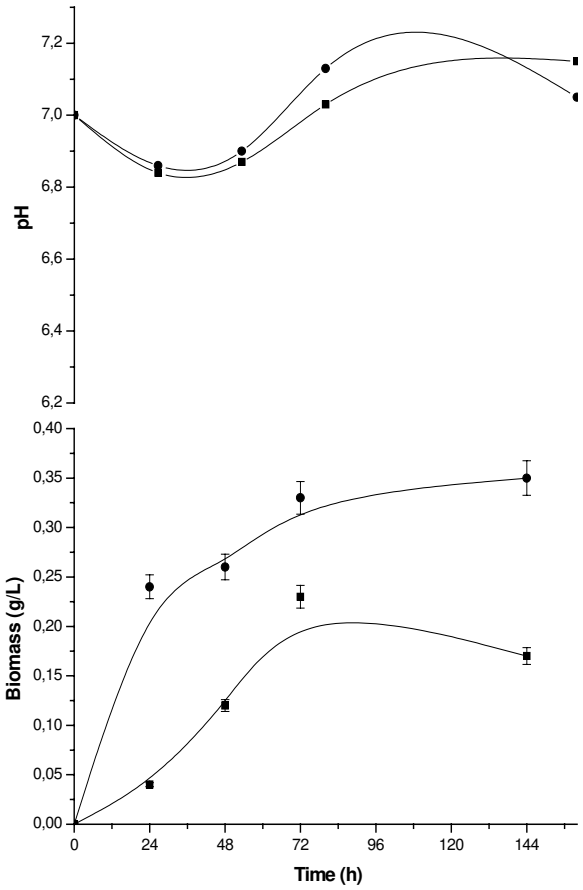

Figure 1. Growth and pH of *Pseudomonas fluorescens* cultured in Luria Bertani medium with 2 mM dibenzothiophene (DBT) as sole sulfur source. (_●_) pH with DBT; (_■_)pH

Table1. Values of μ_{esp} and T_G during kinetics growth of *P. fluorescens*

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

Utilization of DBT by Pseudomonas 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. The time course of DBT utilization by *Pseudomonas fluorescens* is shown in Figure 2 and registers a decrease in the amount of DBT. Biodegradation started after 48 hours of culture, presenting a decrease on the initial DBT concentration from 2 mM to 1.98 mM, and at the end of fermentation fell to a concentration of 0.54 mM, metabolizing about 73% of DBT.

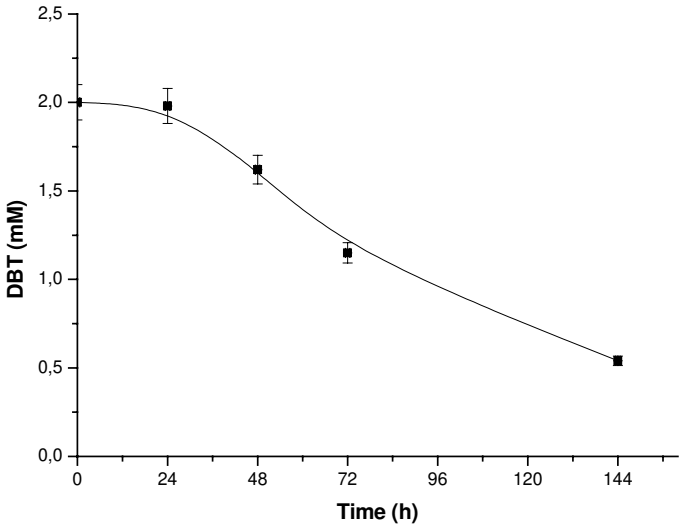

Figure 2. Time course of dibenzothiophene (DBT) utilization by *Pseudomonas fluorescens* (UCP 1514A)cultured in Luria Bertani medium with 2 mM DBT as sole sulfur source.

Desulfurization of DBT by pseudomonas fluorescens and determining pathway intermediates

In order to determine the pathway intermediates of DBT degradation by *Pseudomonas fluorescens*, GC-MS of ethyl acetate extracts of cultures grown over different periods was performed. GC-MS analysis of the extracts revealed several peaks (Figures 4-6). Besides the solvent peaks, 3 peaks were further analyzed by their mass spectra to deduce the structures (Table 2).

Table 2. Mass spectral characteristics of proposed degradation products

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]oxatiin 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]oxatiin 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

As shown in Figures 4-6, three compounds were detected as metabolites in addition to DBT (16.7 min, *m/z* = 184); one metabolite was identified as benzoic acid (11.9 min, *m/z* = 105)*;* a 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 deduced from Figure 3 and the DBT-desulfurizing pathway (Furuya et al, 2001), we can consider that this result suggests that DBT (1) degradation by *P.fluorescens* started from dibenzothiophene sulfone, with

further degradation to benzoic acid and changes from methanecarbothiolic acid to 2-mercaptobenzoic acid (2).

According to Jong-Su et al, (2006) the 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 identified as dibenzothiophene sulfone (Table 2, Figure 3).

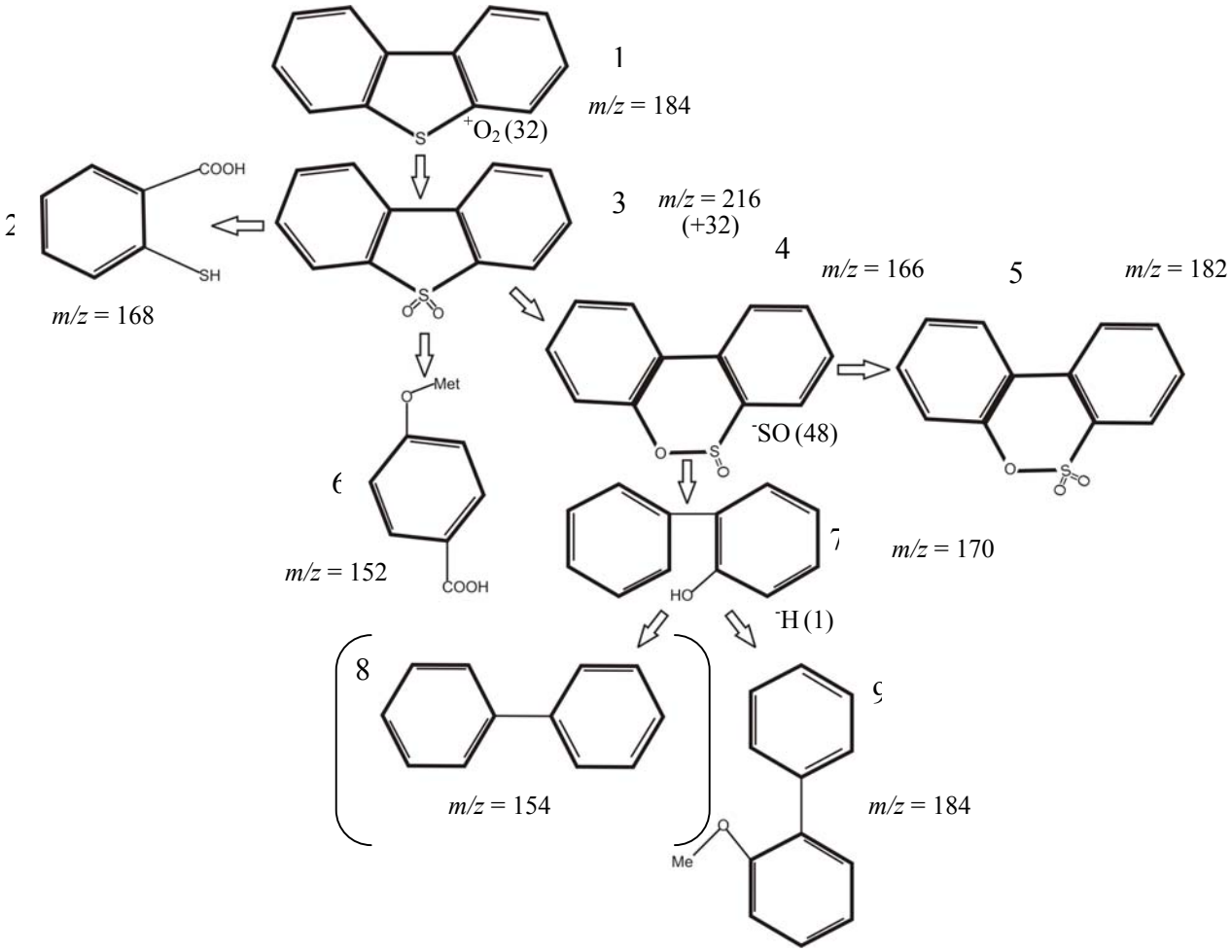

Figure 3. Proposed catabolic pathways of dibenzothiophene by *Pseudomonas fluorescens* (UCP 1514A). (1), Dibenzothiophene; (2), 2-Mercaptobenzoic acid (Me); (3), Dibenzothiophene 5,5'-dioxide; (4), dibenzo(e)[1,2]oxathiin S-oxide; (5), dibenzo(c) [1,2]oxathiin S,S-dioxide; (6), 4-methoxybenzoic acid;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

Detection of dibenzothiophene sulfone demonstrates the presence of the step of sulfur oxidation. According to Konishi et al, (2000), the metabolite (4) was assumed to be dibenzo[c] [1,2]oxatiin S-oxide (*m/z* =166). The abundance of fragment ion 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]oxatiin S,S-dioxide. Dibenzothiophene was transformed to dibenzothiophene sulfone (Bezalel et al, 1996; Ichinose et al, 2002) 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 Li et al, (2005) the metabolite (7) was assumed to be 2-HBP, (*m/z* = 170); the 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.

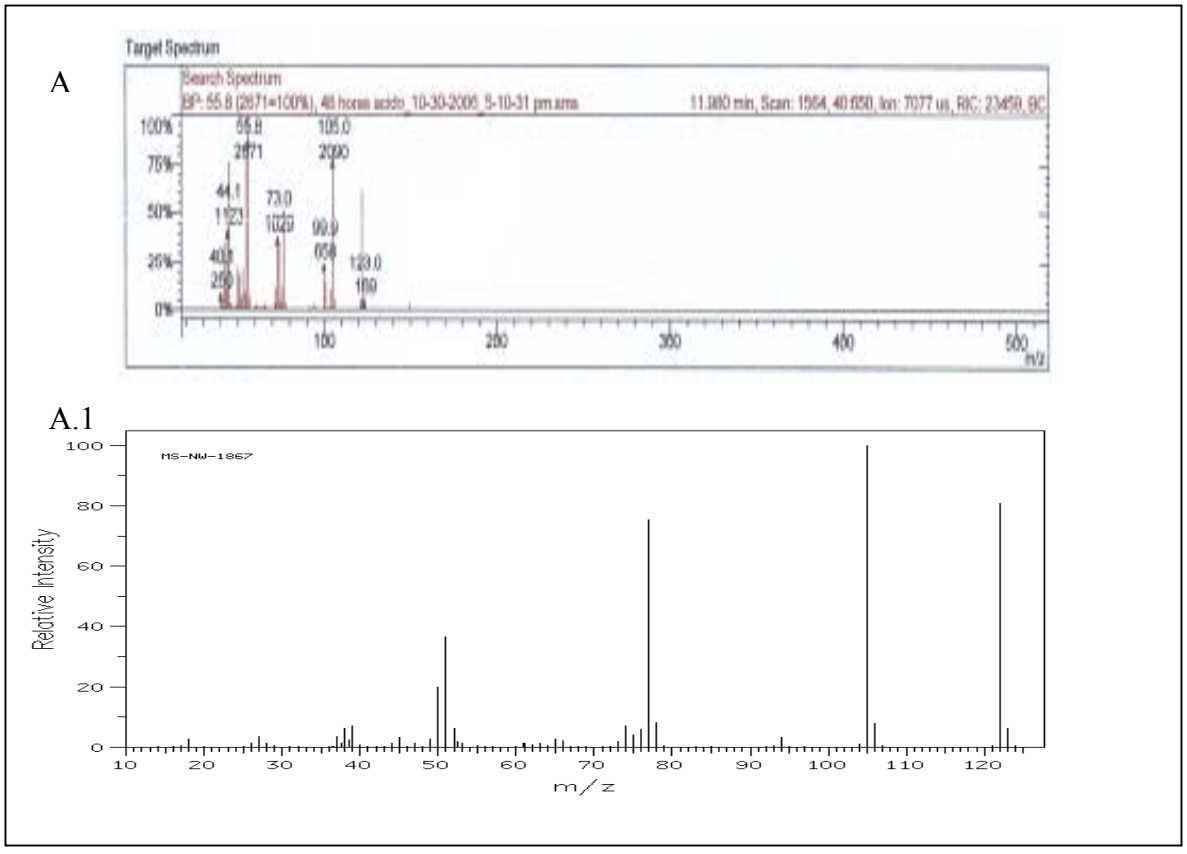

Figure 4. Representative mass spectra of dibenzothiophene in *Pseudomonas fluorescens.* A, Benzoic acid; A.1, Benzoic acid (Spectral Database for Organic Compounds SDBS library.

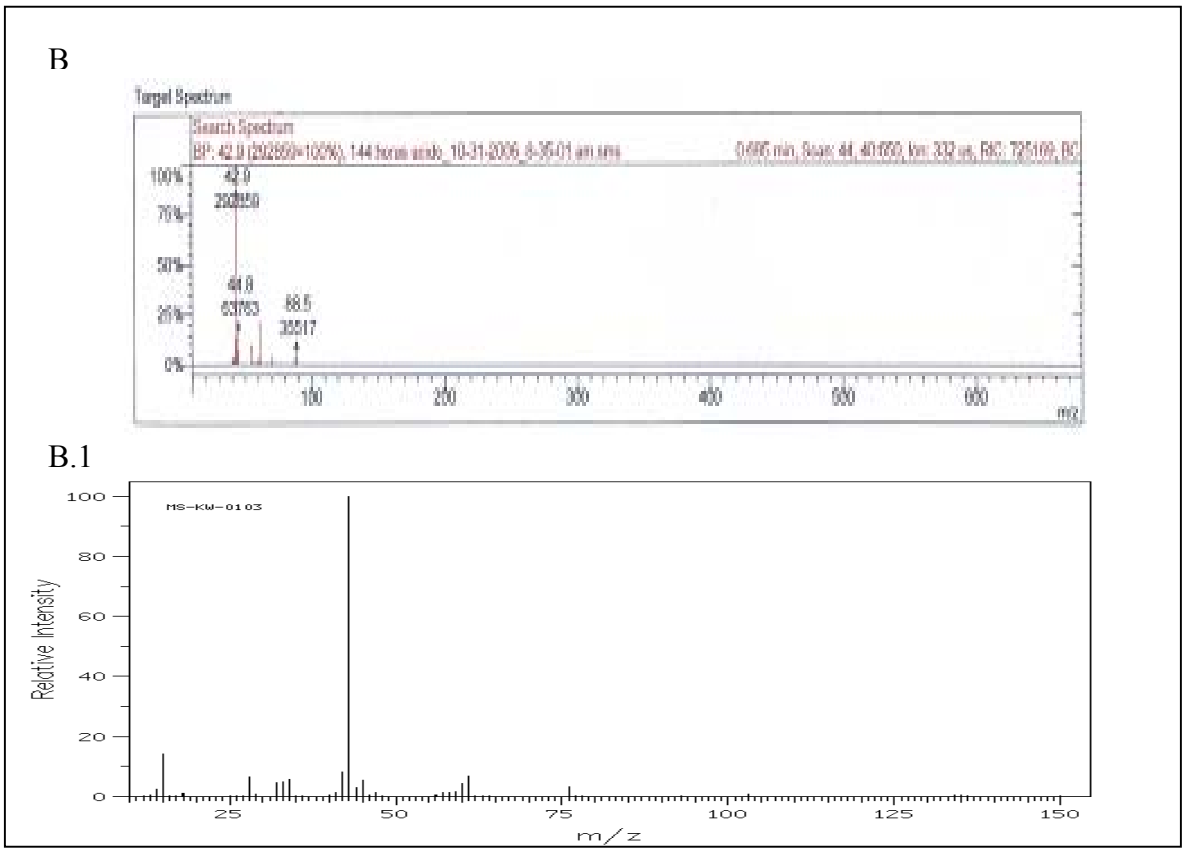

Figure 5. Representative mass spectra of dibenzothiophene in *Pseudomonas fluorescens.* B, methanecarbothiolic acid; B.1, methanecarbothiolic acid (Spectral Database for Organic Compounds SDBS library.

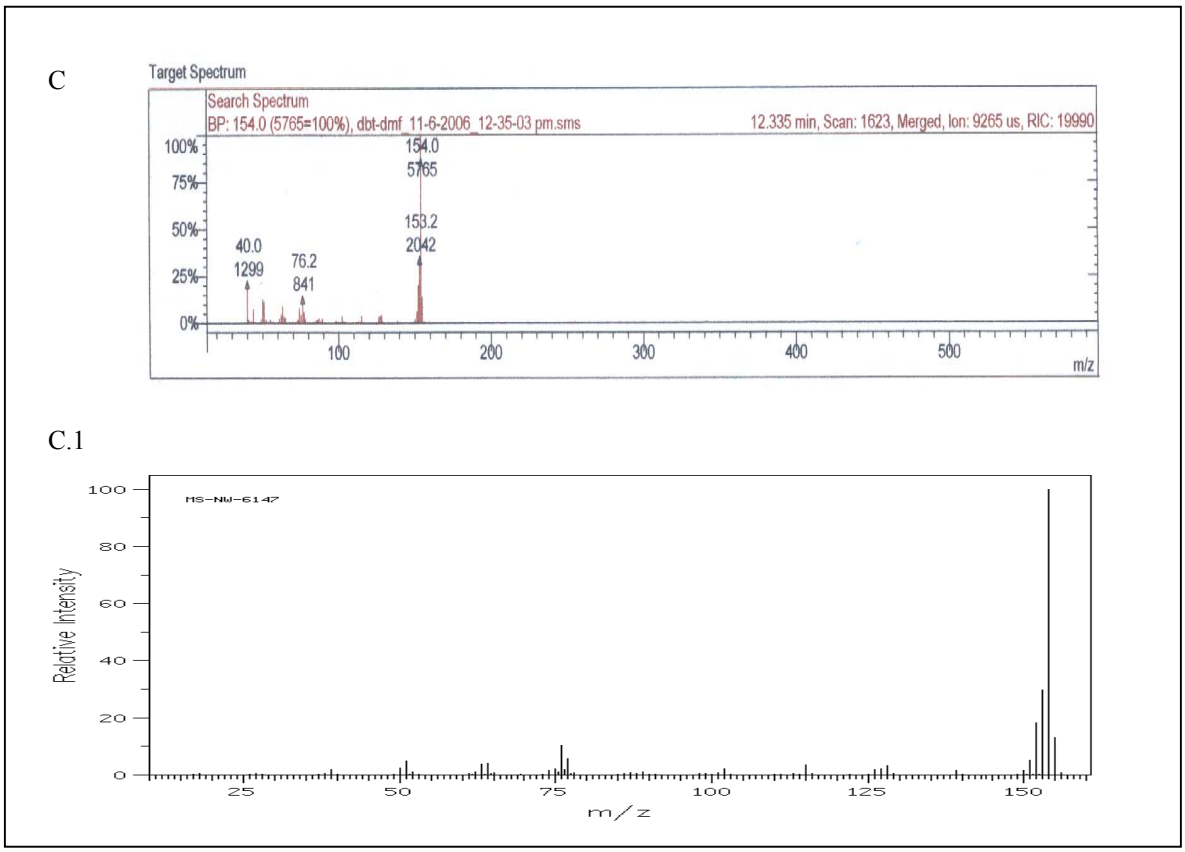

Figure 6. Representative mass spectra of dibenzothiophene in *Pseudomonas fluorescens.* C, Biphenyl; C.1, Biphenyl (Spectral Database for Organic Compounds SDBS library.

Discussion

P. fluorescens UCP 1514A remained viable in the presence of DBT, probably due to this element being considered essential for the formation of aminoacids such as cisteyne, cistyne, methionine, some vitamins and other compounds important for the survival of microorganisms (Castro et al, 2000; Izumi et al, 2001*;* Kirimura et al, 2001).

The development of bio-desulfurization has been prompted by increasingly stringent regulations on the sulfur content in transportation fuels.

The BDS is based on 4S pathway. Through the pathway, DBT is not degraded, but transformed into 2-HBP partitions in the oil phase, resulting in no loss of caloric value of the fuel (Mohebali et al, 2007). DBT-containing hydrocarbon was desulfurized by growing cells of *Pseudomonas* sp. (Setti et al, 1995), as well as by growing and resting cells of *Gordona* sp. (Rhee et al, 1998; Chang et al, 2000) at 30°C.

Setti et al, 1992 reported on n-alkane compounds in a deasphaltenated heavy oil. Several authors stated that the initial degradations of anthracene, dibenzothiophene or pyrene in living cultures of lignolytic fungi do not depend on lignolytic activity, and suggested that cytochrome P-450 monooxygenase could be responsible for this initial step (Bezalel et al, 1996; Gramss et al, 1999; Verdin et al, 2004).

Catabolic pathway of DBT was proposed, based on the metabolites tentatively identified by their mass spectra (Figure 3). The analysis of DBT metabolites suggested that *Pseudomonas fluorescens* UCP(1514A) can decompose DBT through multiple pathways. Seo et al, (2006) reported dibenzothiophene 5-5'-dioxide, which is involved in the sulfur specific process of bio-desulfurization named as 4S pathway (Oldfield et al, 1997).

In this study, DBT degradation by *Pseudomonas fluorescens* started from dibenzothiophene sulfone which was probably further metabolized to 2-mercaptobenzoic acid, according to Seo et al, (2006). Finkelstein et al, (1997) detected several DBT metabolites including benzo[*b*]thiophene-2,3-diol, 2-mercaptobenzoic acid, and 2,2'-dithiosalicylic acid from *Pseudomonas fluorescens* 17 and 26 cultures.

According to Eibes et al, (2006), dibenzothiophene was transformed to dibenzothiophene sulfone and metabolized to 4-methoxybenzoic acid, a ring cleavage product. Konishi et al, 2000 reported further degradation of benzothiophene S-oxide to dibenzo[c][1,2]oxatiin S-oxide and Dibenzo[c][1,2]oxatiin S,S- dioxide. No peak corresponding to dibenzothiophene sulfone was detected in our analysis. However, it is likely that dibenzothiophene sulfone is also an intermediate compound in the bacterial dibenzothiophene degradation pathway.

Conversion of dibenzothiophene S,Sdioxide into dibenzo[c][1,2]oxathiin S-oxide could be explained by the oxidative cleavage of one of the two C-S bonds in dibenzothiophene S,S-dioxide and circularization of the cleavage product under acidic conditions. Benzo[c][1,2]oxathiin S-oxide was also found as the metabolite from benzothiophene in *Gordonia* sp. 213E strain (Gilbert et al, 1998).

This can be formed from (Z)-2-(2'-hydroxyphenyl)ethene1-sulfinate, the thiophene ring-open form, under acidic extraction conditions (Konishi et al, 2000). As shown in Figure 3, two possible metabolites, considered not to include any sulfur in their molecular structures, were presented at the end of the biodegradation pathway of dibenzothiophene.

Based on the deduced structures such as 2-MBP and biphenyl, it is interesting to note that *P. fluorescens* 1514A 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 (Li et al, 2003) These results suggest that the production of 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 UCP1514A 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 (Ohshiro et al, 1996).

In bio-desulfurization, DBT is converted to 2-HBP, which increases the possibility of environmental pollution (Ichinose et al, 1999).

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, $DBTO_2$ and HPBS were not detected by GC-MS analysis.

Although we do not have a clear explanation for this, it might be due to the lability of desulfurized DBT metabolites (Furuya et al, 2001) or the existence of an additional degradation pathway(s) for DBT (Kropp et al, 1998).

Therefore, UCP1514A 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 ways.

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

On ODS, sulfur-containing hydrocarbons can be only oxidized to sulfoxides and sulfones using H_2O_2 as oxidants in acetonitrile as solvents (Chica et al, 2006). Desulfurization can be performed efficiently, through irradiation combined with chemical catalyst. But according to Qu et al, (2006), the results indicated that the combination of H_2O_2-HAC and γ radiation could remove DBT efficiently. However, the consumption of chemicals (H_2O_2 and HAC) seemed to be excessive, which would result in a higher cost.

Until now, conventional refining processes have been performed at much higher temperatures, therefore thermophilic biodesulfurization is desirable and could be easily integrated into the refining process without cooling the stock to 30°C (Konishi et al, 1997).

Moreover, thermophilic biodesulfurization also reduces the viscosity of crude oil, which makes the development of crude oil biodesulfurization more practicable (Borgne and Quintero, 2003). It can be concluded that the physiologic mutant of *Pseudomonas fluorescens* 1514A offer good potential for use in the biocatalytic desulfurization of fossil fuels. Further work aimed at developing the strain is underway.

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CAPÍTULO IV

CONCLUSÕES GERAIS

7. CONCLUSÕES GERAIS

- A linhagem de *Pseudomonas fluorescens* UCP 1514A apresentou resistência à alta concentração de DBT, revelando uma mutação fisiológica em seu perfil genético, sugerindo um alto potencial nos processos de dessulfurização de combustíveis fósseis.

- Com relação à biodegradação do DBT, observa-se que *Pseudomonas fluorescens* apresentou uma rápida adaptação ao meio, ocorrendo o processo de degradação durante às 48 horas de cultivo. O composto é reduzido no processo final da fermentação, em 73%, em relação à concentração inicial.

- As condições experimentais apresentadas neste trabalho revelam a natureza dinâmica desses processos metabólicos. E ainda, permitem o entendimento e caracterização da cinética de crescimento do microrganismo durante a mineralização de compostos organossulfurados.

- Durante o processo de dessulfurização, *Pseudomonas fluorescens* metabolizou o dibenzotiofeno, apresentando como produto final da via de degradação, o bifenil, que pode ser utilizado nos combustíveis fósseis sem que o valor energético do mesmo seja perdido, além de diminuir a poluição ambiental causada pela combustão dos derivados de petróleo.

- De acordo com os resultados conclui-se que *Pseudomonas fluorescens* oferece grande potencial na biodegradação de compostos sulfurados, podendo ser utilizada como catalisador biológico na desssulfurização e mineralização desses compostos, consequentemente, minimizando os danos ambientais causados pela poluição dessas substâncias.

8. ADENDO

Durante o decorrer deste trabalho auxiliamos em outras pesquisas e seus resultados foram submetidos para publicação e apresentados em congressos:

SILVA, T.A.L.; TAMBOURGI, E.B.; CAMPOS-TAKAKI, G.M. **Influence of dibenzothiophene on the growth of *Pseudomonas fluorescens* and its potential in the biodesulfurization,** II Brazilian Symposium on Petroleum Biotechnology Old and New Energy Sources, Universidade Federal do Rio Grande do Norte –UFRN e realizado pelo CROB/LBMG do DBG-CB/UFRN, Natal, 13 à 17 de novembro de 2006.

SILVA, T.A.L.; ARAÚJO, H.W.C.; FREITAS SILVA, M.C.; TAMBOURGI, E.B.; CEBALLOS, B.S.O.; CAMPOS-TAKAKI, G.M. **Produção de biossurfactante por *Pseudomonas fluorescens* utilizando petróleo e óleo diesel como substratos,** X Encontro Nacional de Microbiologia Ambiental, Goiânia, 28 de novembro à 01 de dezembro de 2006.

SILVA, T.A.L.; TAMBOURGI, E.B.; CAMPOS-TAKAKI, G.M. **Potencial de *Pseudomonas fluorescens* na biodegradação de dibenzotiofeno associado à minimizaçào das contaminações ambientais,** Prêmio Petrobrás de Tecnologia edição 2006/2007.

PROCESS BIOCHEMISTRY

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Full length articles (FLA) should not generally exceed 25 double-spaced pages of text (not including the references) and should not contain more than 15 figures and/or tables.

Short communications (SCO) should not exceed 10 double-spaced pages of text (not including the references) and no more than 5 figures and/or tables.

Reviews (REV) should not generally exceed 20 double-spaced pages of text (not including the references) and should not contain more than 10 figures and/or tables.

Accelerated publications can sometimes be taken into consideration. The authors should then clearly motivate the reasons of the accelerated way in the cover letter.

Each paper should be provided with an abstract of 100-150 words reporting concisely on the purposes and results of the paper, and also six keywords.The title of the paper should unambiguously reflect its contents. Where the title exceeds 70 characters a suggestion for an abbreviated running title should be given.

The SI system should be used for all scientific and laboratory data: if, in certain instances, it

is necessary to quote other units, these should be added in parentheses. Temperatures should be given in degrees Celsius. The unit 'billion' (109 in America, 1012 in Europe) is ambiguous and should not be used. Abbreviations for units should follow the suggestions of the British Standards publication BS 1991. The full stop should not be included in abbreviations, e.g. m (not m.), ppm (not p.p.m.), % and / should be used in preference to 'per cent' and 'per'. Where abbreviations are likely to cause ambiguity or may not be readily understood by an international readership, units should be put in full.

Footnotes should be avoided especially if they contain information which could equally well be included in the text. The use of proprietary names should be avoided. Papers essentially of an advertising nature will not be accepted.

Colour illustrations in the print version are reproduced at the author's expense. The publisher will provide the author with a cost estimate upon receipt of the accepted paper. Colour illustrations in the online version are always at no cost to the authors.

References: References should be cited at the appropriate point in the text by a number in square brackets. A list of references, in numerical order, should appear at the end of the paper. All references in this list should be indicated at some point in the text and vice versa. Unpublished data or private communications **AND WEBSITE ADDRESSES** should not appear in the list. Examples of layout of references are given below.

[1] Hsieh C, Hsu TH, Yang FC. Production of polysaccharides of Ganoderma lucidum (CCRC36021) by reusing thin stillage. Process Biochem 2005;40:909-916.

[2] Stephanopoulos GN, Aristidou AA, Nielsen JE. Metabolic engineering: principles and methodologies. New York: Academic Press; 1998. p. 494

[3] Zhong JJ, Yoshida T. Rheological chracteristics of suspended cultures of Perilla frutescens and their implications in bioreactor operation for anthocyanin production. In: Ryu DDY, Furusaki S editors. Advances in Plant Biotechnology. Amsterdam: Elsevier Science; 1994. p. 255-279.

[4] Lima R, Salcedo, RL. An optimized strategy for equation-oriented global optimization. In: Grievink J, Schijndel JV. editors. 10th European Symposium on Computer Aided Chemical Engineering. New York: Academic Press; 2002. p. 913-918.

[5] Curtin CD. Towards molecular bioprocessing as a tool to enhance production of anthocyanins in Vitis vinifera L. cell suspension culture. Australia: Flinders University; Ph.D. thesis; 2004. p.250.

[6] Snow-Brand-Milk-Prod. Lysozyme purification by affinity chromatorgraphy on crosslink chitosan sulfate. Jpn. Patent. JP 05260-966. 92.03.24.

[7] Enfors SO, editor. Physiological stress responses in bioprocesses. Advances in Biochemical Engineering/Biotechnology. vol. 89. Berlin: Springer; 2004. p. 244.

[8] Schweder T, Hecker M. Monitoring of stress response, In: Enfors SO, editor. Physiological stress responses in bioprocesses. Advances in Biochemical Engineering/Biotechnology vol. 89. Berlin: Springer; 2004. p. 47-71.

The ISME Journal

Guide for Authors

Welcome to the electronic manuscript submission website for *The ISME Journal*. The instructions below are structured so you can quickly and easily answer the following questions:

1. Is my manuscript suitable for *The ISME Journal*? (Aims and Scope)
2. How do I format my manuscript for *The ISME Journal*? (Format of Papers)
3. How do I submit my manuscript to *The ISME Journal*? (Submission of Papers)

Aims and Scope

The ISME Journal seeks to promote diverse and integrated areas of microbial ecology spanning the breadth of microbial life, including bacteria, archaea, microbial eukaryotes, and viruses. Contributions of broad biological interest and impact are especially encouraged. Topics of particular interest within the journal's scope include those listed below:

Microbial population and community ecology

- Theoretical advances in microbial population and community ecology, including novel theoretical development relevant to the diversity and structure of microbial populations and communities, advances in modeling and comparisons of microbial ecological principles with those in macroecology
- Biogeography of microbial populations
- Environmental factors (biotic and abiotic) defining the distribution and abundance of microbial populations
- Integrated advances in microbial ecophysiology
- Phage genetics and ecology and environmental virology, including studies of interactions between viruses and the environment, vectors of viral transmission, epidemiology, and diversity (including generation and maintenance)
- Community level research of microbial assemblages, with emphasis on the contribution of individuals and populations
- Microbial survival and persistence mechanisms: Development and selection for resistance (heavy metals, antibiotics etc.)

Microbe-microbe and microbe-host interactions

- Microbial communication and signaling, and advances that allow study on scales relevant to microbial interactivities
- Plant-microbe interactions, including feedback and response pathways, underlying mechanisms, environmental cues, unique traits, evolution, adaptation and fitness
- Threat of emerging diseases (pathogenicity, epidemiology, ecology of reservoirs, vectors and host)
- Symbioses and syntrophic relationships
- Microbial contribution to medical biotechnology and microbial therapy
- Commensal microbial ecology – intestinal, oral, etc.

Evolutionary genetics

- Ecological aspects of experimental evolution
- Insights into genome evolution and adaptation
- Genetics and ecology of the horizontal gene pool
- Advances in mathematical and evolutionary genetics

Integrated genomics and post-genomics approaches in microbial ecology

- Studies of in situ function, gene regulation and expression
- Metagenomic genomic approaches to understanding and accessing the genomic potential of microbial communities
- Novel microbial ecology approaches involving (environmental) proteomics and metabolomics
- Theoretical and practical advances in Bioinformatics, including improved linkages between ecological parameters and molecular data, as well as advances in curation and annotation practices
- Novel "-omics' approaches that address microbial activities and potential at the single-cell level

Microbial engineering

Environmental Biotechnology, including ecological interactions key to waste water treatment, water management, biofilters, energy production, etc.

- Development and mechanisms of microbial biocatalysts
- Developments in bioremediation and biodegradation
- Microbial contributions and potential in biofuel technologies
- Microbial process modeling and its application

Geomicrobiology and microbial contributions to geochemical cycles

- Integrated advances in biogeochemistry
- Microbial contributions to geochemical cycles
- Importance and mechanisms of microbe-mineral interactions

Microbial ecology and functional diversity of natural habitats

- Terrestrial and subsurface microbial ecology
- Aquatic and sediment microbial ecology
- Linking phylogeny and function in diverse ecosystems – common, novel and extreme
- Biofilm functional microbial ecology
- Aero – microbiology (distribution, source impact, etc), including issues of climate and dispersal
- Microbial processes and interactions in extreme or unusual environments

Microbial ecosystem impacts

- Impacts of microbial processes on climate change, and impacts of climate change on microbial communities and processes
- Food web structure, nutrient flow, and biological transformations from micro- through macro-scales
- Systems microbiology and integration of microbial ecology into systems ecology

Prior to Submission

Editorial Policy

Editors in Chief: Mark Bailey, John Heidelberg, George Kowalchuk.

A manuscript will be considered for publication on the understanding that all named authors have agreed to its submission and that if accepted it will not be later published in the same or similar form in any language without the consent of the publishers. The editors also encourage submission of review articles, short communications and commentaries.

The editors reserve the right to reject manuscripts without review. Such rejections must be approved by all editors-in-chief, and are intended to alleviate unnecessary workload for the editorial board, as well as provide authors the opportunity to seek other publishing options as soon as possible.

To avoid unnecessary delays in the review process, please consider the following policies carefully before you submit your manuscript.

Availability of published material

It is understood that by publishing a paper in The ISME Journal the authors agree to make freely available to colleagues in academic research any of the organisms, viruses, cells, nucleic acids, antibodies, and other reagents that were used in the research reported and that are not available from commercial suppliers.

Conflicts of interest

In the interests of transparency and to help reviewers assess any potential bias, The ISME Journal requires authors of original research papers to declare any competing commercial interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper.

Electronic manipulation of images

Digital image enhancement is acceptable practice, although it can result in the presentation of quite unrepresentative data as well as in the loss of meaningful signals. During manipulation of images a positive relationship between the original data and the resulting electronic image must be maintained. If a figure has been subjected to significant electronic manipulation, the specific nature of the enhancements must be noted in the figure legend or in the 'Materials and methods' section. The Editors reserve the right to request original versions of figures from the authors of a paper under consideration.

Submission to public databases

The ISME Journal will only review and publish manuscripts if the authors agree to make all data that cannot be published in the journal itself (e.g. novel nucleotide sequences, structural data, or data from large-scale gene expression experiments) freely available in one of the public databases (see Submission to public databases below). Accession codes must be provided at the time a revised manuscript is returned to the Editorial Office. To avoid delays in publication of the manuscript, we encourage authors to deposit relevant data in public databases prior to submission. The authors may request that the data be stored in a confidential section of the database, in which they can request passwords from the database administrators, and these should be passed on to the Editorial Office to allow the editors and referees to anonymously access the information during the review process.

Supplementary information for the editors and the reviewers

Any manuscripts under review or accepted for publication elsewhere should accompany the submission if they are relevant to its scientific assessment. Authors should also provide upon submission any kind of supplementary material that will aid the review process.

Content types

The content types accepted by *The ISME Journal* are:

Original article

Short communication

Winogradsky review (by invitation only)

Mini-Review

Commentary

Format of Papers

Preparation of manuscripts

Manuscripts are considered with the understanding that they have not been published previously in print or electronic format and are not under consideration by another publication or electronic medium. Copies of possibly duplicative materials that have been previously published or are being considered elsewhere must be provided at the time of manuscript submission.

Cover Letter

The uploaded covering letter must state that the material has not been submitted for publication elsewhere while under consideration for the *The ISME Journal*. Identify the name, full postal address, and fax number, of corresponding author. The authors are free to offer suggestions of suitable expert reviewers. The layout of the paper should be as follows;

Short Communication

Studies that fall short of the criteria for full research papers (eg preliminary experiments limited by sample size or duration, novel hypotheses or commentaries) may be submitted as Short Communications. They should contain no more than 1000 words of text, a maximum of two display items (tables and/or figures) and a maximum of 20 references. Apart from the Abstract (one paragraph of maximum 150 words) Keywords and Subject Category, there is no obligation to divide the text into sections. In all other respects, the directions for full papers should be followed.

Commentary

Commentaries are meant to discuss issues of particular significance to the field of microbial ecology. Commentaries may include highlights of significant papers, in the current issue or elsewhere, or comprise poignant opinions, responses to previously published items, or other timely information or comment. Commentaries should not exceed 900/1500 words and have 10 or fewer references. Commentaries may be either solicited by the editors or offered as an unsolicited submission. If you wish to offer an unsolicited commentary contribution, we ask you to first contact the editorial office with your request, including a short description of the content and implications of your commentary.

Winogradsky Review (only by invitation of Editors)

Word limit of 5,000 words including abstract but excluding references, tables and figures. Winogradsky reviews are a comprehensive analysis of specific topics in microbial ecology that are solicited by the Editors. Proposals for Reviews may be submitted; however, in this case authors should only send an outline of the proposed paper for initial consideration. Charges for color images will be negotiated with *The ISME Journal*.

Mini-Review

Word limit of 3,000 words including abstract but excluding references, tables and figures. A number of mini-reviews will be solicited by the editors, however we also welcome timely, unsolicited mini-reviews. Authors with proposals for mini-reviews, should present information concerning the proposed content and authors of their mini-review to the editors prior to submission. Unless otherwise informed, all changes for color images will be the authors' responsibility.

Original Articles

Type preferably on A4 paper (210 x 297 mm) single-sided and double-spaced with 25 mm margins. Number each page following the title page and include line numbers (every 5 lines is sufficient) if this option is available. Please make spelling consistent with current editions of either Webster's Dictionary or Oxford English Dictionary.

Manuscripts should be divided in to the following sections:

Title page

Abstract

Introduction

Materials and methods

Results

Discussion

Acknowledgements

References

Figure legends

Figures

Tables

Supplementary Information

Title page

Title page, giving a concise but informative title, and the first and last names, and other initials, and affiliations (but not degrees) of all contributors (formerly called authors). The order in which the contributors are listed should be agreed amongst the investigators, and should indicate that the first listed made the greatest contribution to the paper. Please provide a running title of no more than 50 characters including spaces.

Three to six keywords, which may or may not appear in the title, should be given in alphabetical order, below the abstract, each separated by a slash (/).

Subject Categories are used to structure the current and archived online content of The ISME Journal, and to help readers interested in particular areas of microbial ecology find relevant information more easily. Subject Categories are also indicated in the table of contents and on the title page of the published article.

Authors should suggest an appropriate Subject Category for the submitted manuscript. One category may be selected from the following list:

- Microbial population and community ecology
- Microbe-microbe and microbe-host interactions
- Evolutionary genetics
- Integrated genomics and post-genomics approaches in microbial ecology
- Microbial engineering
- Geomicrobiology and microbial contributions to geochemical cycles
- Microbial ecology and functional diversity of natural habitats
- Microbial ecosystem impacts

Abstract An abstract of not more than 250 words. The Abstract should be comprehensible to readers before they have read the paper, and abbreviations and reference citations within the abstract should be avoided.

Introduction This should give a short, clear account of the background and reasons for undertaking the study. It should not be a review of the literature.

Materials and methods This section should contain sufficient detail so that all experimental procedures can be repeated by others, in conjunction with cited references. This section may be divided into subheadings to assist the reader. Extended protocol details can be submitted as supplementary information.

Results The description of results should not simply reiterate data that appear in tables and figures and, likewise, the same data should not be displayed in both tables and figures. The results section should be concise and follow a logical sequence. If the paper describes a complex series of experiments, it is permissible to explain the protocol/experimental design

before presenting the results. Do not discuss the results or draw any conclusions in this section. This section may be divided into subheadings to assist the reader. Large datasets or other cumbersome data pertinent to the manuscript may be submitted as supplementary information.

Discussion Do not recapitulate the results, but discuss their significance against the background of existing knowledge, and identify clearly those aspects that are novel. The final paragraph should highlight the main conclusion(s), and provide some indication of the direction future research should take. This section may be divided into subheadings to assist the reader. Results and Discussion may be combined.

Acknowledgments These should be brief, and should include sources of support, sources of material (eg novel compounds, strains, etc.) not available commercially.

Units and Abbreviations Try to restrict the use of abbreviations to SI symbols and those recommended by the IUPAC. Abbreviations should be defined in brackets after their first mention in the text, not in a list of abbreviations. Standard units of measurements (SI symbols) and symbols of chemical elements may be used without definition in the body of the paper. Abbreviations of standard biochemical compounds, e.g. ATP, DNA, nucleotides in nucleic acids, and amino acids in proteins, need not be defined.

References

Authors are responsible for the accuracy of the references. Published articles as well as those in press (please state the name of the journal and enclose a copy of the manuscript) may be included. In the text of the manuscript, a reference should be cited by author and year of publication eg (Bailey & Kowalchuk, 2006) and (Heidelberg et al, 1994) and listed at the end of the paper in alphabetical order of first author. References should be listed and journal titles abbreviated according to the style used by Index Medicus, examples are given below. All authors should be quoted for papers with up to six authors; for papers with more than seven authors, the first six only should be quoted, followed by *et al*.

Example of journal references:

1. Cho JC, Kim MW, Lee DH, Kim SJ (1997) Response of bacterial communities to changes in composition of extracellular organic carbon from phytoplankton in Daechung reservoir (Korea). *Arch. Hydrobiol*. **138**:559-576

Example of book chapter:

Zinder, SH (1998) Methanogens. In: Burlage, RS (Ed.) *Techniques in Microbial Ecology*. Oxford University Press, Oxford, **pp 113-136**.

Example of book:

Sambrook J, Fritsch E & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Press*, Cold Spring Harbour, New York, USA

Citations to articles in press or only published online at the time of submission should be made as follows:

Example of article in press without doi:

Lim E-K, Ashford DA, Hou B, Jackson RG, Bowles DJ (2004) *Arabidopsis glycosyltransferases as biocatalysts in fermentation for regioselective synthesis of diverse quercetin glucosides*. Biotech Bioeng, (in press)

Example of article in press with doi

Eng-Kiat Lim and Dianna J Bowles, *A class of plant glycosyltransferases involved in cellular homeostasis*, The EMBO Journal advance online publication 8 July 2004; doi: 10.1038/sj.emboj.7600295

Figures

Figures and images should be labeled sequentially, numbered and cited in the text. Figure legends should be printed, double spaced, on a separate sheet titled 'Titles and legends to figures'. Figures should be referred to specifically in the text of the paper but should not be embedded within the text. The use of three-dimensional histograms is strongly discouraged when the addition of the third dimension gives no extra information. If a table or figure has been published before, the authors must obtain written permission to reproduce the material in both print and electronic formats from the copyright owner and submit it with the manuscript. This follows for quotes, illustrations and other materials taken from previously published works not in the public domain. The original source should be cited in the figure caption or table footnote. Colour figures can be reproduced if necessary, but the authors will be expected to contribute towards the cost of publication. A quote will be supplied upon acceptance of your paper.

Artwork Guidelines

Detailed guidelines for submitting artwork can be found by downloading the guidelines PDF. Using the guidelines, please submit production quality artwork with your initial online submission. If you have followed the guidelines, we will not require the artwork to be resubmitted following the peer-review process, if your paper is accepted for publication.

Colour on the web

Authors who wish their articles to have FREE colour figures on the web (only available in the HTML (full text) version of manuscripts) must supply separate files in the following format. These files should be submitted as supplementary information and authors are asked to mention they would like colour figures on the web in their submission letter.

For Single Images:

Width	**500 pixels** (authors should select "constrain proportions", or equivalent instructions, to allow the application to set the correct height automatically.)
Resolution	**125 dpi** (dots per inch) or "Save for Web" if using Photoshop
Format	**JPEG** for photographs **GIF** for line drawings or charts
Filenaming	Please save image with .jpg or .gif extension to ensure it can be read by all platforms and graphics packages.

For Multi-part Images :

Width	**900 pixels** (authors should select "constrain proportions", or equivalent instructions, to allow the application to set the correct height automatically.)
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Format	**JPEG** for photographs **GIF** for line drawings or charts
Filenaming	Please save image with .jpg or .gif extension to ensure it can be read by all platforms and graphics packages.

Authors may be asked to pay the full colour fee for figures that are not submitted in the format described above.

Tables

These should be labeled sequentially as Table 1, Table 2, etc. **Each table should be saved in a separate file**, numbered and titled, and cited in the text. Reference to table footnotes should be made by means of Arabic numerals. Tables should not duplicate the content of the text. They should consist of at least two columns; columns should always have headings. Authors should ensure that the data in the tables are consistent with those cited in the relevant places in the text, totals add up correctly, and percentages have been calculated correctly. Unlike figures or images, tables may be embedded into the word processing software if necessary, or supplied as separate electronic files.

House Style

As the electronic submission will provide the basic material for typesetting, it is important that papers are prepared in the general editorial style of the journal.

1. See the artwork guidelines for information on labeling of figures
2. Do not make rules thinner than 1pt (0.36mm)
3. Use a coarse hatching pattern rather than shading for tints in graphs
4. Color should be distinct when being used as an identifying tool
5. Use SI units throughout
6. Spaces, not commas should be used to separate thousands
7. Abbreviations should be preceded by the words for which they stand in the first instance of use
8. Text should be double spacing with a wide margin
9. At first mention of a manufacturer the town, (state if USA) and country should be provided.

File Formats:

File formats for manuscript files, figures and tables that are acceptable for our electronic manuscript submission process are given on the online forms. Further advice on file types is also available from the Tips webpage. Please follow our artwork guidelines for submitting figures, and use a common word-processing package (such as Microsoft Word) for the text. Either embed tables converted into images at the end of your Word document, or as a separate files in which ever program you used to generate them. If you submit raw data, this can be done in Excel, or tab/comma delimited format.

Saving files with Microsoft Office 2007

Microsoft Office 2007 saves files in an XML format by default (file extensions .docx, .pptx and xlsx). Files saved in this format cannot be accepted for publication.

Save Word documents using the file extension .doc

- Select the Office Button in the upper left corner of the Word 2007 Window and choose "Save As"
- Select "Word 97-2003 Document"
- Enter a file name and select "Save"

These instructions also apply for the new versions of Excel and PowerPoint.

Equations in Word must be created using Equation Editor 3.0

Equations created using the new equation editor in Word 2007 and saved as a "Word 97-2003 Document" (.doc) are converted to graphics and can no longer be edited. To insert or change an equation with the previous equation editor:

- Select "Object" on the "Text" section of the "Insert" tab
- In the drop-down menu - select "Equation Editor 3.0"

Do not use the "Equation" button in the "Symbols" section of the "Insert" tab.