UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

Production of *Gordonia alkanivorans* strain 1B biomass in bioreactor and further application towards fossil fuels desulfurization

Mestrado em Microbiologia Aplicada

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2015

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This thesis was fully performed at the Department of Bioenergy of Laboratório Nacional de Energia e Geologia (LNEG) under the direct supervision of Dr. Luís Alves in the scope of the *Master in Applied Microbiology* of the Faculty of Sciences of the University of Lisbon.

Prof. Dr. Ana Reis was the internal designated supervisor in the scope of the *Master in Applied Microbiology* of the Faculty of Sciences of the University of Lisbon

"In a dark place we find ourselves… and a little more knowledge might light our way."

- Yoda in *Star Wars: Episode III Revenge of the Sith*

Agradecimentos

Quero deixar um agradecimento a todos aqueles que ajudaram (direta ou indiretamente) à elaboração desta dissertação e sem os quais este trabalho não teria sido possível.

Ao Doutor Luís Alves e à Doutora Susana Paixão Alves por terem aceitado ser meus orientadores e por me terem acolhido como estagiária numa altura em que muitos outros não o fariam. O vosso apoio, compreensão e paciência foram fundamentais para levar este trabalho a bom porto. Por tudo isto um grande obrigado.

À Professora Doutora Ana Reis, pelos conselhos e disponibilidade para me coorientar (mesmo após uma mudança radical de tema de dissertação). Muito obrigada por todo o apoio.

À Professora Doutora Lélia Chambel, por toda a disponibilidade demonstrada para resolver os problemas que foram surgindo no decurso deste trabalho e por me ter aceitado neste mestrado.

Ao grupo de colegas, funcionários e investigadores do Laboratório Nacional de Energia e Geologia (LNEG). Ao Engenheiro Carlos Barata por toda a ajuda e ensinamentos no que toca ao funcionamento do laboratório e na solução de vários problemas. A todos os meus colegas, Doutor Tiago Lopes, Engenheira Margarida Gonçalves, Dra. Patrícia Branco, Dra. Catarina Figueiredo e Doutora Maria José, muito obrigada pela amizade, companhia e apoio nas mais diversas situações. Ao Engenheiro Diogo Sebastião por todas as tertúlias que nos proporciona e por me obrigar a ir almoçar e lanchar a horas decentes. Um obrigado muito especial à Dra. Ana Sofia Fernandes, que comigo e com o Dr. Tiago Silva formou uma equipa de trabalho excecional. Muito obrigada pela companhia em todos aqueles fins-de-semana de trabalho e noitadas a filtrar tupinambo e acima de tudo pela tua amizade, apoio e compreensão quando as coisas se tornaram mais difíceis (e muito obrigada pelos maravilhosos jantares!).

Ao meu querido grupo de amigos que já não se encontram no LNEG, Engenheiro Francisco Mendes, por fazer sobremesas deliciosas com farinha, ao Dr. Bruno Arez, por todas as "suecadas" e noitadas, à Dra. Diana Francisco, Dr. João Caldeira, Engenheiro Vasco Martins muito obrigada pela vossa amizade e apoio.

Um grande obrigado há minha querida e favorita Engenheira Joana Ortigueira (à qual nem sei muito bem como agradecer). Muito obrigada por seres uma companhia constante, por me ligares todos os dias, por me desviares para jogar contigo, por ouvires os meus desabafos, por me ajudares com as revisões da tese e por me mimares com cheesecake de Nutella e After Eights quando estava mais em baixo. No fundo muito obrigada por existires e seres minha amiga.

Muito obrigada aos meus "afilhados" da FCUL, Dr. Flávio Ferreira, Dr. João Messias, Dra. Teresa Santos, Dr. Pedro Henrique, Dr. Gonçalo Rodrigues, peço desculpa não ter estado tão presente como

Resumo

A população mundial tem vindo a crescer exponencialmente nos últimos 100 anos, maioritariamente nos países em desenvolvimento como a China ou a India. Estas populações continuam a usar o petróleo como fonte primária de combustível, esperando-se por isso um aumento da procura de 11,25% até 2035 (OPEC 2013). A descoberta da tecnologia necessária para a extração de crude de zonas de difícil perfuração revolucionou o mercado da energia, diminuindo o preço do barril de petróleo até valores históricos (U.S Energy Informaton Administration 2014). Contudo, crudes extraídos destes novos poços possuem na sua composição grandes quantidades de compostos de enxofre e outros contaminantes cuja remoção é extremamente complicada, sendo designados "crudes pesados". A combustão dos petróleos e derivados leva à libertação destes compostos, para a atmosfera, podendo causar diversos problemas ambientais e de saúde. Desta forma, é imperativa a sua remoção, sendo que diversos países, tais como os Estados Unidos da América, a China e os países pertencentes à União Europeia, começaram a legislar de modo a restringirem a quantidade de enxofre presente nos produtos petrolíferos, exigindo a criação do chamado "ultra-low sulfur oil" (OPEC 2014).

Hoje em dia, o processo utilizado nas refinarias para a remoção de enxofre dos crudes é a hidrodessulfurização (HDS). Este processo consiste na utilização de altas temperaturas e pressões e catalisadores de última geração para remover os compostos de enxofre. Contudo, compostos como o dibenzotiofeno (DBT), que são os grandes contribuidores para o enxofre presente nos crudes, são altamente recalcitrantes a este processo, uma vez que a molécula de enxofre se encontra rodeada por dois anéis benzénicos. Para a remoção destes compostos, a indústria desenvolveu a HDS profunda. Esta utiliza temperaturas e pressões mais elevadas que a HDS e catalisadores mais eficientes sendo também menos ecológica e mais dispendiosa (Klein 1999).

Existe no entanto uma alternativa biológica à HDS profunda, a biodessulfurização (BDS). Esta é um processo biotecnológico equivalente à HDS, uma vez que remove o enxofre de compostos complexos. Nas últimas décadas, a BDS tem vindo a atrair atenções, visto que tem a capacidade de processar os combustíveis fósseis de uma forma amiga do ambiente. Esta dá-se a temperaturas e pressões iguais às ambientais e é efetuada por microrganismos que têm a capacidade de metabolizar compostos de enxofre (Soleimani *et al.* 2007; Alves & Paixão 2011).

Neste estudo foi utilizada a bactéria *Gordonia alkanivorans* estirpe 1B. As espécies do género *Gordonia* são bastante atrativas para usos biotecnológicos, uma vez que, têm a capacidade de degradar inúmeros poluentes ambientais, tais como hidrocarbonetos policíclicos aromáticos, alquilpiridinas, xenobióticos ou polímeros naturais. Têm também a capacidade de transformar ou sintetizar compostos orgânicos (transformação de esteroides e produção de carotenoides) *(*Linos *et al.* 1999; Harner *et al.* 2011; Grace Liu *et al.* 2011). A estirpe 1B já tinha sido descrita como tendo a

capacidade de converter DBT em 2-HBP (2-hidroxibifenil) por Alves *et al.* (2005). Foi igualmente demonstrado, por Alves (2007), que a bactéria em questão possui, à semelhança de *Rodococcus erythropolis* (uma espécie até então muito utilizada em ensaios de dessulfurização), um operão *dsz*, composto pelos genes *dsz*A, *dsz*B e *dsz*C que codificam as proteínas necessárias para que a célula consiga dessulfurizar. Alves & Paixão (2014b) identificaram também outra característica metabólica interessante na estirpe 1B, nomeadamente o fato de esta crescer preferencialmente utilizando frutose como fonte de carbono.

Este comportamento é raro em bactérias, contudo abriu uma nova linha de investigação em que se deu prioridade à pesquisa de fontes de carbono alternativas, ricas em frutose (nomeadamente resíduos agroindustriais), tais como melaço de cana, medronho, alfarroba ou tubérculos de tupinambo (Silva 2012; Silva *et al.* 2012; Alves & Paixão 2014a; Silva *et al.* 2015). Todas estas fontes de carbono alternativas produziram melhores resultados de dessulfurização quando comparadas com fontes ricas em glucose, contudo, o sumo de tupinambo (JAJ) foi o melhor, de entre as fontes alternativas ricas em frutose testadas (Silva 2012). Os tubérculos de tupinambo são ricos em inulina, um frutano linear composto por um número variável de moléculas de frutose unidas por ligações β (2 →1) com uma molécula de glucose no final, ligada à cadeia de frutoses por uma ligação α (1 → 2). A inulina é solúvel em água e pode ser facilmente hidrolisada (através de hidrólise ácida ou enzimática), libertando frutose (até 95%) e glucose (Silva 2012; Paixão *et al.* 2013). Por estas razões, o JAJ foi escolhido como a fonte de carbono alternativa a ser utilizada neste trabalho.

Sendo que a BDS é mais eficiente e menos dispendiosa que a HDS profunda, pode ser utilizada nas refinarias como um complemento à HDS de maneira a produzir combustíveis com muito baixo teor de enxofre (Alves *et al.* 2015). Quando implementada, esta técnica vai diminuir os problemas ambientais levantados pela HDS, reduzindo a emissão de CO_2, a produção de resíduos e o consumo de energia. Tudo isto se traduz em menos capital investido (dois terços do capital necessário para a HDS) e menos custos operacionais (Vazquez-Duhalt *et al.* 2002; Alves *et al.* 2015). O processo de BDS pode ainda beneficiar da produção simultânea de produtos de alto valor acrescentado, que podem ser valorizados através de outros processos industriais (De Miguel *et al.* 2000; De Miguel *et al.* 2001; Veiga-Crespo *et al.* 2012; Bandyopadhyay & Chowdhury 2014).

Este trabalho, teve como objetivo principal a minimização do meio de cultura utilizado para o crescimento da bactéria *Gordonia alkanivorans* estirpe 1B (meio mínimo sem sulfatos/meio SFM). Para isso, avaliou-se quais os componentes do meio mais importantes para o crescimento e desenvolvimento celular, tendo sido selecionados para minimização a fonte de azoto (NH_4Cl), a fonte de magnésio ($MgCl_2.6H_2O$) e a solução de micronutrientes (TES), primeiro em frasco agitado e de seguida em cultura em contínuo.

O azoto (N) é um grande interveniente no crescimento celular e um dos nutrientes necessários à vida como a conhecemos. Faz parte da composição de aminoácidos, ácidos nucleicos e enzimas/coenzimas e no meio mínimo utilizado para crescer a bactéria *G. alkanivorans* é o nutriente utilizado em maior concentração (1,22 g.l^{-1}), sem contar com a fonte de carbono (Singh 1971; Alves *et al.* 2015). Portanto, para minimizar a quantidade de fonte de azoto foram testadas diversas formulações do SFM com concentrações decrescentes de NH_4Cl (F#1 – 100%, F#2 – 75%, F#3 – 50% e F#4 – 25%). Os resultados obtidos mostraram que o crescimento foi grandemente afetado tanto com F#4 como com F#3. Nestes dois casos não houve consumo total dos açúcares fornecidos, provando que a quantidade de azoto era insuficiente. Contudo, com F#2, a curva de crescimento foi semelhante à produzida pelo controlo F#1, ocorrendo o consumo total dos açúcares.

No que toca à dessulfurização, quando crescida com a F#1, a cultura conseguiu obter uma taxa máxima específica de produção de 2-HBP (q_{2-HBP}) de 2,5 µmol.g^{-1}(peso seco).h^{-1}, com consumo total do DBT fornecido. Mas, com a diminuição da concentração de azoto, a q_{2-HBP} foi também decrescendo, atingindo um valor de 2,4 µmol.g^{-1}(peso seco).h^{-1} no crescimento com F#2; 2,3 µmol.g^{-1}(peso seco).h^{-1} no crescimento com F#3; e 1,4 µmol.g^{-1}(peso seco).h^{-1} no crescimento com F#4. Nestas três condições testadas não ocorreu o consumo total do DBT fornecido, indicando que a concentração de azoto utilizada não foi suficiente. Contudo, o facto de o crescimento ter ocorrido normalmente com a F#2 indica que esta está muito próxima da concentração mínima necessária à célula, portanto 75% <fonte de N mínima <100%.

Quanto ao magnésio (Mg), este foi escolhido como um dos elementos do meio a minimizar pois é essencial à divisão celular e é um interveniente especialmente importante para bactérias grampositivas, uma vez que faz parte da estrutura do peptidoglicano (Webb 1939). Ao testar-se concentrações decrescentes de $MgCl_2.6H_2O$ (F#1 – 100%; F#5 – 75%; F#6 – 50%; F#7 – 25%), foi possível observar que não houve alterações significativas nem no perfil de crescimento (sendo a absorvância a 600 nm (OD_{600nm}) semelhante para todas as formulações testadas), nem no consumo de açúcares. Contudo, em termos de dessulfurização, verificou-se um comportamento interessante. A diminuição da concentração de magnésio levou a um aumento significativo da q_{2-HBP} de 2,5 µmol.g^{-1}(peso seco).h^{-1} (F#1) para 2,8 µmol.g^{-1}(peso seco).h^{-1} (F#5); 3,0 µmol.g^{-1}(peso seco).h^{-1} (F#6); e 3,4 µmol.g^{-1}(peso seco).h^{-1} (F#7). Este resultado indica que o magnésio pode inibir, de certa forma, a dessulfurização, de tal modo que a diminuição da concentração utilizada faz com que esta aumente significativamente.

O último componente do meio testado foi a solução de micronutrientes (TES). Esta solução é composta por minerais como ferro, zinco, molibdénio, manganésio ou cobalto (Alves & Paixão 2014b). Estes micronutrientes fazem parte da estrutura das proteínas celulares e funcionam muitas vezes como cofatores (Majzlik *et al.* 2011; Kirsch & Eitinger 2014). Neste caso, as concentrações

testadas anteriormente (F#8 – 75%, F#9 – 50% e F#10 – 25%) não foram suficientes para se observar diferenças quer no crescimento, quer no consumo de açúcares, quer em termos de dessulfurização. Testaram-se então concentrações mais baixas, nomeadamente, 5% (F#11), 2,5% (F#12) e 0% (F#13). Com estas concentrações já foi possível observar alterações nos parâmetros avaliados. Com F#11, as células foram capazes de consumir toda a fonte de carbono fornecida, contudo, só o fizeram após 118 horas de crescimento. Relativamente às restantes concentrações, verificou-se que não houve conclusão do crescimento visto não ter ocorrido o consumo total da fonte de carbono. Quanto à dessulfurização, o consumo total do DBT foi atingido em todos os frascos, exceto na ausência de TES. Para F#8, F#9 e F#10, a $q_{2\text{-HBP}}$ foi mais elevada que o controlo, sendo que nas restantes concentrações testadas foi quase duas vezes mais baixa.

A informação obtida nos diferentes ensaios em frasco agitado permitiu a minimização das concentrações dos três componentes essenciais presentes na formulação original do meio, sem afetar negativamente o crescimento celular e ainda melhorando a dessulfurização. Estes resultados permitiram desenvolver o meio SFM minimizado, ao qual se denominou "meio SFMM", consistindo em 85% N (estipulado através de uma extrapolação baseada nos resultados do consumo de açúcares e dessulfurização), 25% Mg e 25% TES. De seguida, o meio SFMM e o meio SFM foram testados com duas fontes de carbono distintas: frutose + glucose e JAJ precipitado (JAJ$_p$). Em geral, estes ensaios demostraram que, independentemente da fonte de carbono utilizada, a utilização do meio minimizado (meio SFMM) aumentou a dessulfurização pela estirpe 1B, em comparação com o respetivo controlo (meio SFM).

Este facto foi de encontro ao esperado, uma vez que a minimização do Mg e do TES fez com que a $q_{2\text{-HBP}}$ fosse mais elevada. A estirpe 1B foi capaz de crescer normalmente no meio minimizado e não foram observados efeitos de sinergia negativos entre os compostos do meio. Comparando os resultados por fonte de carbono é possível observar, no entanto, que para ambos os meios (SFM e SFMM), as taxas máximas de crescimento (μ_{max}) e a dessulfurização ($q_{2\text{-HBP}}$) foram mais baixas quando o JAJ$_p$ foi utilizado como fonte de carbono. Isto pode ser o resultado de um efeito inibitório causado pelo cloreto de bário utilizado durante a precipitação dos sulfatos.

Para a BDS ser integrada nas refinarias, a produção de biomassa deverá ocorrer num quimiostato trabalhando em modo contínuo (Pacheco 1999). Este sistema tem a vantagem de permitir o controlo total sobre diversos parâmetros da cultura, tais como a temperatura, a agitação, o pH, o arejamento e a velocidade a que o meio de cultura entra no quimiostato. Tendo isto em conta, o passo seguinte consistiu na adaptação do SFMM (anteriormente otimizado em frasco agitado) para uso em quimiostato. Sendo que este trabalho tem como base a minimização de custos do processo, foi igualmente desenvolvido um quimiostato de baixo custo. Neste, foi efetuado um "screening" à

concentração de azoto, uma vez que tinha ficado estipulado no ensaio de frasco que a concentração ótima seria um valor entre 75-100%.

Tal como foi observado no ensaio em frasco agitado, os parâmetros analisados foram melhores com o aumento da concentração de azoto. Ensaios de citometria de fluxo foram efetuados de modo a perceber o estado fisiológico das células crescidas com as diferentes concentrações de azoto. Foi possível observar que com menos de 100% de azoto, a percentagem de células com a membrana comprometida aumenta, sendo que as percentagens de células metabolicamente inativas ou mortas mantêm-se constantes. Um dos parâmetros que pode ser utilizado para calcular a concentração ótima de NH_4Cl é a taxa de produção de biomassa (BPR). Fazendo uma regressão polinomial da BPR = f (concentração de N) foi possível interpolar que 90% seria a concentração mais acertada, uma vez que o valor da BPR foi muito semelhante ao obtido com o 100%.

Desenhado o meio SFMM para quimiostato (meio $SFMM_R$), consistindo em 90% N, 25% Mg e 25% TES, este foi testado com duas fontes de carbono: frutose + glucose *versus* JAJ para a produção de biocatalizadores. A taxa de diluição foi de 0,065 h^{-1} para crescimentos com fontes comerciais e 0,045 h^{-1} para crescimentos com JAJ (fonte alternativa de carbono), com base nos resultados previamente obtidos em frasco agitado. Comparando os dados metabólicos das duas culturas em estado estacionário SS#5 e SS#6 (SS#5 – $SFMM_R$ + (Frutose + Glucose); SS#6 – $SFMM_R$ + JAJ concentrado/JAJ_c), é possível observar que a cultura SS#5 apresenta uma produção de biomassa (3,97 $g.l^{-1}$) superior à da cultura SS#6 (3,10 $g.l^{-1}$), sendo estes resultados consistentes com os restantes parâmetros metabólicos associados ao crescimento (OD_{600nm}, biomassa, taxa de produção de biomassa, taxas de consumo de açúcares, q_{Fru}, q_{Glu}). Contudo, esta diferença de 1,7 vezes pode estar associada às diferenças na taxa de diluição, sendo que, no SS#5 foi 0,065 h^{-1} e no SS#6 foi de 0,045 h^{-1} o que pode levar à produção de células metabolicamente mais ativas. Quanto aos resultados obtidos para a recuperação de carbono (CR), a eficiência da conversão de carbono (CCE) e o rendimento celular ($Y_{x/s}$), verificou-se que são muito semelhantes para ambas as culturas, demonstrando um comportamento similar das células crescidas no meio minimizado, apesar da utilização de uma fonte de carbono diferente. O meio $SFMM_R$ também permitiu o consumo total da fonte de carbono (e por consequência de todos os nutrientes do meio) em ambas as culturas SS, permitindo obter um $q_{2\text{-HBP}}$ de 2,90 $\mu mol.g^{-1}$(peso seco).h^{-1} para o SS#5 e um $q_{2\text{-HBP}}$ de 12,23 $\mu mol.g^{-1}$(peso seco).h^{-1} para o SS#6, sendo este último 4 vezes superior ao obtido com o meio $SFMM_R$ com a fonte de carbono comercial (Frutose + Glucose).

Os resultados obtidos recorrendo à citometria de fluxo demonstraram que em ambos os SS, as células se encontram maioritariamente saudáveis, sendo a percentagem de células viáveis aproximadamente 93% tanto para a cultura SS#5, como para a cultura SS#6. Desta forma, os resultados metabólicos e fisiológicos apontam para as células da cultura SS#6 ($SFMM_R$ + JAJ_c) como

sendo os biocatalizadores otimizados e mais eficientes em termos de custos e em termos de produtividade (biodessulfurização). A taxa de dessulfurização obtida com este meio foi igualmente 2,4 vezes superior à obtida por Silva (2012) utilizando células da estirpe 1B crescidas em JAJ_p ($q_{2\text{-HBP}} = 5,06\ \mu mol.g^{-1}$(peso seco)$.h^{-1}$).

Para melhor compreender as diferenças observadas em termos de dessulfurização, utilizou-se a técnica de PCR em tempo real para analisar a expressão dos genes *dsz*A, *dsz*B e *dsz*C. Analisando os resultados observou-se uma grande diminuição na expressão dos genes *dsz*B e *dsz*A no caso da cultura SS#5 ($SFMM_R$ + Frutose + Glucose), em comparação com a cultura SS#6 ($SFMM_R$ + JAJ_c). Estes resultados podem explicar a diferença entre taxas de dessulfurização, uma vez que a expressão dos genes necessários à via não está a ocorrer na mesma proporção.

Numa refinaria, as células de *G. alkanivorans* estirpe 1B vão ter que dessulfurizar petróleo, que é rico em diferentes espécies de tiofenos (onde está incluído o DBT). À escala laboratorial, o crude pode ser mimetizado utilizando uma mistura de tiofenos dissolvidos num solvente orgânico (combustível modelo). Desta forma testou-se o desempenho de células provenientes do quimiostato, crescidas com $SFMM_R$ + JAJ_c (cultura SS#6), a dessulfurizar um combustível modelo. Os resultados obtidos indicam que as células foram capazes de dessulfurizar 500 µM de uma mistura de DBT e derivados de DBT em apenas 72 h, sendo que ao final deste tempo já só são observáveis os produtos da dessulfurização.

De modo a viabilizar economicamente todo este processo é necessário aproveitar produtos secundários que a célula possa produzir. No caso da bactéria utlizada neste estudo, durante o crescimento, são produzidos pigmentos (entre outros produtos secundários) que podem ser aproveitados após a dessulfurização. Neste contexto, os pigmentos produzidos pela estirpe 1B foram extraídos de células de ambos os crescimentos em quimiostato (culturas SS#5 e SS#6), antes e após a dessulfurização. Após a sua identificação em HPLC, observou-se que os pigmentos obtidos são principalmente da família dos carotenoides, sendo que dos vários picos observados, houve correspondência para a astaxantina, luteína e cantaxantina. Os resultados obtidos demonstram que as células exaustas (SS#5-postBDS; SS#6-postBDS) produziram maiores quantidades dos carotenoides identificados (luteína, astaxantina e cantaxantina) do que as células frescas (SS#5-preBDS; SS#6-postBDS). A luteína foi o carotenoide mais abundante, tanto para as células pré-dessulfurização como para as células pós-dessulfurização de ambos os estados estacionários. Estes resultados também demonstraram um perfil de carotenoides diferente para as células produzidas no SS#5 e no SS#6. As células produzidas no SS#5-postBDS atingiram uma produção total de pigmentos de 265 µg.g^{-1}(peso seco), enquanto que as células produzidas no SS#6 obtiveram uma produção total de pigmentos de 334 µg.g^{-1}(peso seco). Como a dessulfurização do DBT melhorou a produção generalizada de todos os pigmentos, a exploração destes produtos de alto

valor acrescentado a partir das células exaustas pode estar associada ao processo de biodessulfurização, como um fator determinante ao seu equilíbrio económico (Paixão *et al.* 2016).

A biodessulfurização é um passo importante para tornar o processo de tratamento de crudes um pouco mais ecológico e amigo do ambiente. Este trabalho permitiu a redução dos componentes do meio de cultura necessário para o crescimento da estirpe 1B e levando ao aumento da capacidade de dessulfurização dos biocatalizadores utilizados na biodessulfurização de combustíveis fósseis e à redução do custo associado à sua produção.

Palavras-chave: Biodessulfurização; enxofre; petróleo; *Gordonia alkanivorans* estirpe 1B; minimização de meios de cultura; bio-reator.

Abstract

Biodesulfurization is an eco-friendly process for the production of ultra-low sulfur fuels. Optimization studies towards the integration of this technology in a petroleum refinery are an important focus of research. The main goal of this study consisted on the minimization of the sulfur free mineral (SFM) medium for the maximum production of efficient desulfurizing biocatalysts (*Gordonia alkanivorans* active cells) taking into account the lowest operational costs. In this context, a series of assays, first in shake-flask and then in chemostat, were carried out to develop and optimize a culture medium containing minimal amounts of Nitrogen and Magnesium sources and TES (trace elements solution). The shake-flask minimization assays allowed the design of a SFMM (SFM minimum) medium containing 75-100% Nitrogen source, 25% Magnesium source and 25% TES, which permitted enhanced desulfurization in comparison with control medium (SFM). This combined reduction was then applied to the SFM reactor medium and the culture conditions were optimized in chemostat assays. After screening for the minimal amount of Nitrogen source using a low-cost bioreactor, the established $SFMM_R$ (SFMM reactor) medium was 90% NH_4Cl, 25% $MgCl_2$ and 25% TES. This $SFMM_R$ medium was tested in two additional continuous cultures and adjusted in order to obtain the maximum yield of biocatalysts (strain 1B resting cells). The use of Jerusalem artichoke juice concentrate as the single carbon source caused a 4-fold increase in desulfurization capability of the biocatalysts (maximum specific 2-hidroxybiphenyl production rate = 12.2 $\mu mol.g^{-1}$(dry cell weight).h^{-1}). These results were consistent with the Real Time-PCR analysis, which showed a higher expression of the overall desulfurization genes (*dsz*A, *dsz*B, *dsz*C) in these biocatalysts. Furthermore, these biocatalysts were also capable of total desulfurization of a model oil, with the exhausted biomass producing 334 $\mu g.g^{-1}$(dry cell weight) of high added-value carotenoids, highlighting their potential towards a cost-effective industrial scale-up.

Key-words: Biodesulfurization; sulfur; crude oil; *Gordonia alkanivorans* strain 1B; culture media minimization; bioreactor.

Abbreviation list

2-HBP – 2-Hydroxybiphenyl

atm – Atmosphere

BDS – Biodesulfurization

CCE – Carbon Conversion Efficiency

C-content – Carbon Content

cDNA – Complementary Deoxyribonucleic Acid

CFDA – 5,6-carboxyfluorescein diacetate

CR – Carbon Recovery (percentage of supplied carbon that is used in the cell constitution)

DBT – Dibenzothiophene

DBTO – Dibenzothiophene 5-oxide

DBTO$_2$ – Dibenzothiophene 5,5-oxide

DCW – Dry Cell Weight

DMF – Dimethylformamide

DMSO – Dimethyl Sulfoxide

DPM – Diesel Particulate Matter

EU – European Union

F# – Formulation Number

Fru – Fructose

Fru+Glu – Fructose + Glucose

g.g^{-1} – gram per gram

GC – Gas Chromatography

GHG – Greenhouse Gas

Glu – Glucose

HDS – Hydrodesulfurization

HPBS – 2-(2'-hydroxyphenyl) benzene sulfinate

HPLC – High Precision Liquid Chromatography

JAJ – Jerusalem artichoke Juice

JAJ$_C$ – Jerusalem artichoke Juice Concentrate

JAJ$_p$ – Sulfate precipitated Jerusalem artichoke juice

LwL – Lower Left Quadrant

LwR – Lower Right Quadrant

mb.d^{-1} – Million Barrels per Day

MEOR – Microbial Enhanced Oil Recovery

Mg-content – Magnesium Content

N-content – Nitrogen Content

OD$_{600nm}$ – Absorbance at 600 nm

ODS – Oxidative Desulfurization

OEDS – Oxidative Desulfurization Combined with Extraction

PCR – Polymerase Chain Reaction

PI – Propidium Iodide

$q_{2\text{-HBP}}$ – Specific Desulfurization Rate

q_{CO2} – Specific Rate of Carbon Dioxide Production

q_{Fru} – Specific Rate of Fructose Consumption

q_{SO4} – Specific Rate of Sulfate Consumption

RC – Resting cells

RNA – Ribonucleic Acid

rpm – Rotations per Minute

RT-PCR – Real Time Polymerase Chain Reaction

S-content – Sulfur Content

SFM – Sulfur Free Mineral

SFMM – Sulfur Free Mineral Minimum

$SFMM_R$ – Sulfur Free Mineral Minimum (Medium Adapted for Bioreactor)

SFM_R – Sulfur Free Mineral (Medium Adapted for Bioreactor)

SS# – Steady-state Culture Number

TES – Trace Element Solution

UpL – Upper Left Quadrant

UpR – Upper Right Quadrant

USA – United States of America

$v.v^{-1}$ – Volume per Volume

vvm – Volume per volume per minute

$w.v^{-1}$ – Weight per Volume

$Y_{x/s}$ – Cell Yield (cell mass produced per mole of substrate consumed)

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1. Introduction

1.1. World energy demand

The worldwide population has been increasing exponentially for the last 100 years from 2 to 7.3 billion and is expected to grow up to 8.6 billion in 2035 (OPEC 2014). Most of this growth is predicted to occur in India, China and other developing countries. As shown in **Figure 1**, these nations have grown economically and financially during the last 35 years, changing from mostly agricultural populations to great urban communities (more than 1.6 billion by 2035).
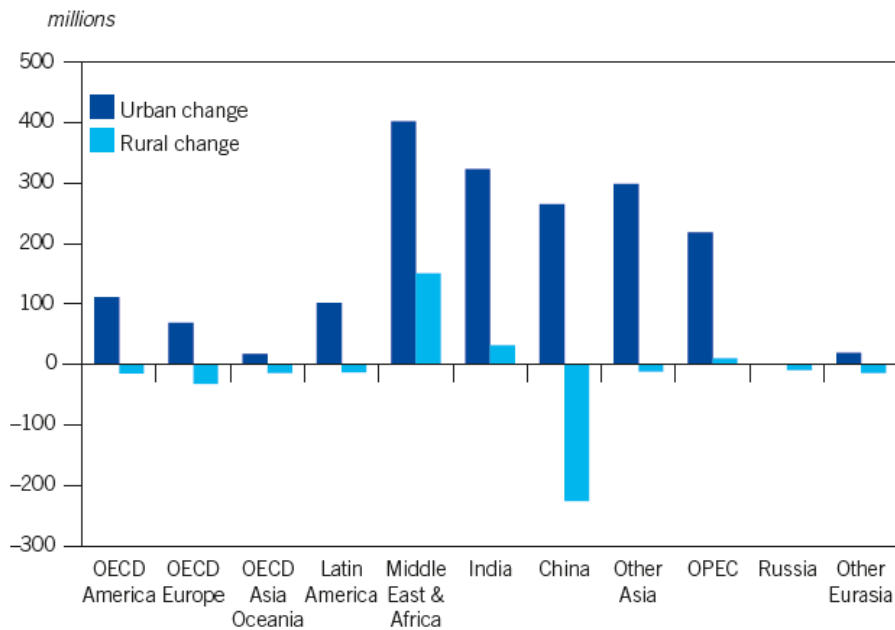
Figure 1 – Evolution of urban and rural population size by region, from 2012 to 2035 (Adapted from World Oil Outlook, 2013, OPEC).

Such changes are leading to higher energy needs to support not only the developed industry but also the highly demanding lifestyles of these populations. Forecasts predict a 52% increase of energy demand from 2010 to 2035 (**Figure 2**). Most of this energy will be of fossil fuel origin (80%), with the largest share, coming from oil (26 to 27%). Oil demand will experience an average increase of 0.9 mb.d^{-1} (million barrels per day) until 2018 and 20 mb.d^{-1} until 2035, which translates in a demand increase of 3.8% and 11.25%, respectively (OPEC 2013).

1.2. The importance of oil

With the emergence of hybrid and non-petroleum-based engines, it was expected that oil consumption would decrease drastically. However, this effect was only observed in developed countries where alternative energy sources are well established and the population economic power allows access to these new technologies. In developing countries the demand for cheaper, reliable and comfortable means of transportation is high, so vehicles with internal combustion engines are the preferred choice.

For example, it is expected that China will have an increase of 380 million vehicles until 2035 (OPEC 2013).
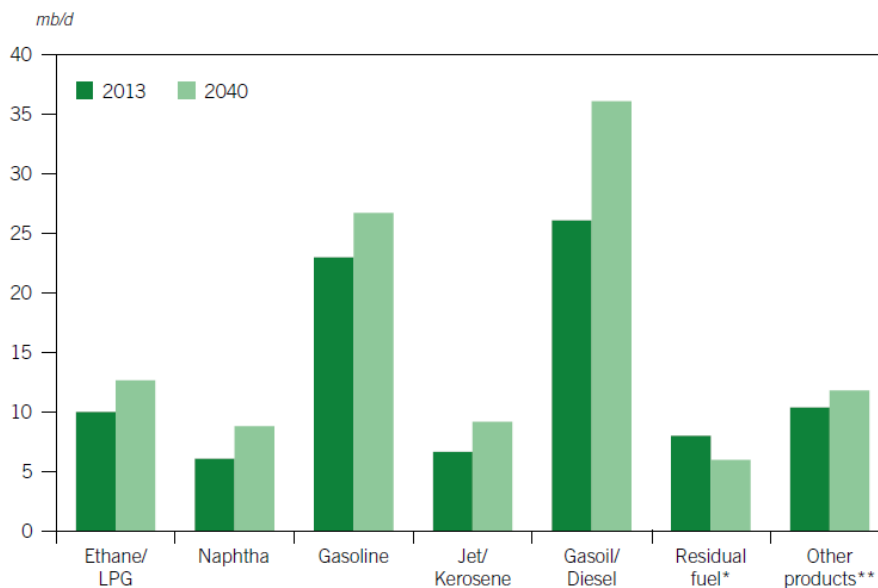
*Figure 2 – Global product demand of 2013 and 2040. *Includes refinery fuel oil; **Includes bitumen, lubricants, petroleum coke, waxes, till gas, sulfur, direct use of crude oil, etc (Adapted from World Oil Outlook, 2014, OPEC).*

Even with its high energy demand, most oil reserves are found in countries where the socio-political scenario is unstable, resulting in great price variation. This fact, coupled with the non-renewable nature of fossil fuel and the consequent depletion of its reserves, has led to a worldwide search for an acceptable replacement (OPEC 2014). Biofuels, such as biodiesel, biogas and bioethanol are considered by many as an alternative. Much research has been conducted throughout the world in the last decades concerning biofuels, however, there are still several drawbacks which limit their short term utility. The competition for resources used in human and animal feed, the fact that their production in high quantities is expensive and laborious and that there are still problems with transport and storing are some of these drawbacks/obstacles (Atabani *et al.* 2012). **Table 1** enumerates a list of more particular problems that these technologies are currently facing.

The discovery of the technology necessary to remove oil from difficult-to-drill sites, such as shale formations or oil sands has revolutionized the energy market bringing oil prices to an historical low. With its relatively cheap operational costs and almost instant investment return, the use of this new technology is resulting in a high flux of oil into the international markets (U.S Energy Informaton Administration 2014; OPEC 2014). Moreover, the use of these shale formations allowed countries that used to be consumers to become producers (U.S Energy Informaton Administration 2014), driving away some of the production from the unstable regions, and greatly increasing the reserves available. Ultimately, these recent changes in the market have made oil re-emerge as a cheap and easily available energy source.

Table 1 – *Disadvantages of some biofuels: Biodiesel, Bioethanol, Biomethane/Biohydrogen (Von Blottnitz & Curran 2007; Bond & Templeton 2011; Atabani et al. 2012; Sánchez-Segado et al. 2012; Lima-Costa et al. 2012)*

Biodiesel	• Has 12% less energy content than diesel, resulting in a 2 to 10% increase of fuel consumption.
	• Has a higher cloud and pour points.
	• Has higher nitrogen oxide emissions than diesel.
	• Has lower volatilities, causing the formation of deposits in engines (due to incomplete combustion characteristics).
	• Causes excessive carbon deposition and gum formation.
	• Has higher viscosity (11–18 times diesel), thus needing higher injector pressure.
	• Its oxidation stability is lower than that of diesel, thus losing calorific power.
	• Produces lower engine speed and power.
	• Has high price, causes high engine wear and engine incompatibility.
	• Requires expensive fatty acid separation or use of less effective (or expensive) acid catalysts.
	• The transesterification has some environmental effects such as waste disposal and water requirement for washing, soap formation, etc.
	• The high temperatures in the transesterification, incomplete conversion and the variability of the incoming feedstock increases the end-product price.
Bioethanol	• The blend of bioethanol with gasoline has to take place right before its use.
	• Is higroscopic (miscible with water), which causes ignition problems.
	• Automobile engines need to be modified to be run on a blend containing 5 (E5, Europe) to 10% (E10, North America) bioethanol.
	• Has over 30% less energy than gasoline, low flame luminosity and lower vapor pressure than gasoline (making cold starts difficult).
	• Low levels of ethanol blended with gasoline increases vapor pressure and favor evaporative emissions that contribute to smog formation.
	• The biomass pre-treatment process has been proven to be unsuitable due to high costs, low yields, produced waste and undesired by-products.
Biomethane/ Biohydrogen	• Has transportation and utilization problems (fuel cell vehicles are not commercially available yet and a distribution infrastructure for hydrogen cannot be assembled in the short term).
	• Has storage problems (biohydrogen has to be compressed, liquefied, or stored in metal hydrides).
	• The production is associated to the generation and emission of CO_2 and N_2O.

1.3. The impact of sulfur in oil quality and environment

The combustion of fossil fuels releases many hazardous components such as CO_2, NO_x, SO_x and other sulfur compounds. Sulfur is a major component of crude oil, along with carbon and hydrogen, ranging from 0.03 to 7.89 % ($g.g^{-1}$), and between 500 and 5000 $mg.l^{-1}$ in the distillate fraction used to make diesel oil (Soleimani *et al.* 2007; Mohebali & Ball 2008). High viscosity and density crudes (such as shale oils) tend to have higher sulfur concentrations (3.09 % ($g.g^{-1}$), especially in its organic forms (e.g. sulfides and thiophenic compounds, represented in **Figure 3**) that can account for 75% of total sulfur. Some of these are very recalcitrant, because the sulfur is enclosed within an aromatic ring. This configuration makes its removal increasingly more difficult.

Due to the presence of sulfur compounds in their composition, the combustion of oils may cause several health and environmental problems. High concentrations of sulfur in diesel oils increase the formation of diesel particulate matter (DPM), which has been classified as carcinogenic by several

regulatory and research agencies, such as the U.S Environmental Protection Agency. DPM related air pollution can also cause bronchial irritation and asthma attacks in susceptible individuals. Thiophene compounds present in fossil fuels, as dibenzothiophene (DBT), have also been proven to be toxic to several species of mammals. The incomplete burning of sulfur rich oil can produce high concentrations of SO_2, which reacts with water in the atmosphere, leading to the formation of sulfuric acid, the main component of acid rains. Sulfur can also be a hindrance to pollution preventing mechanisms, such as catalytic converters seen in automobiles, preventing their complete function and decreasing their efficiency (Lloyd & Cackette 2001; Maricq *et al.* 2002; Saiyasitpanich *et al.* 2005; Mohebali & Ball 2008) .
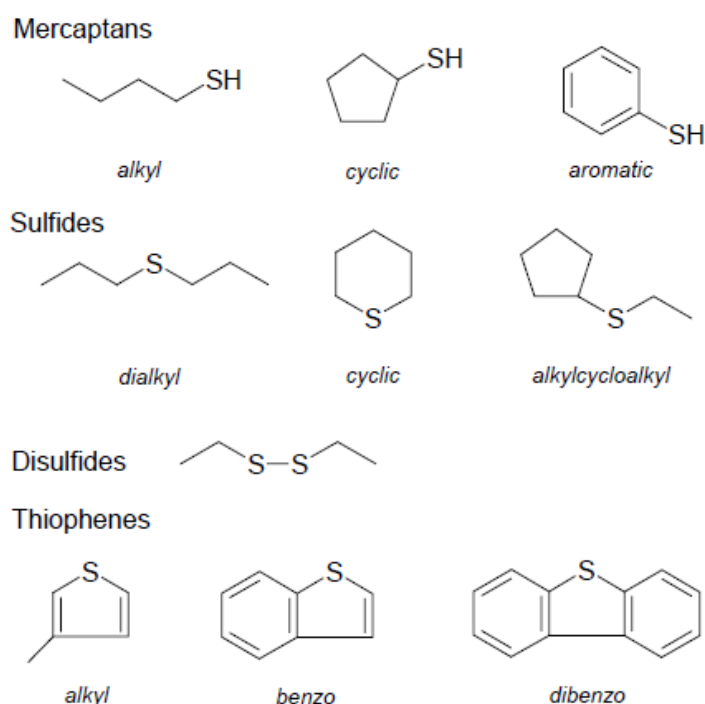
Figure 3 – *Chemical structures of organic sulfur compounds present in crude oils (Adapted from Marcelis, 2002).*

As a response to the increasing concern with the environmental and health problems related with the sulfur levels in fossil fuels, countries have begun to implement strict sulfur concentration limits. In a time span of 7 years, the USA lowered the legal limit from 3400 to 30 ppm for all refineries and, up to 2018, all northeastern states refineries will have adopted <15 ppm of sulfur content oil (U.S Energy Informaton Administration 2014). Since 2009, EU countries have stipulated that all gasoline and on-road diesel must be limited to 10 ppm of sulfur content, and later in 2011 this limit was applied to all types of off-road diesels as well (OPEC 2011). As already stated, China and India are the largest contributors for the world population increase. Confronted with alarming levels of pollution, these countries followed the USA and EU directives and started to implement stricter oil regulations. China has limited the legal sulfur concentration nationwide to 150 ppm, having stricter laws for Beijing, Shanghai, Guangzhou and Shenzhen, where the limit was established at 50 ppm. India has also

lowered its legal limit to 150 ppm sulfur gasoline nationwide and restricted to 50 ppm in 13 selected cities with large vehicle populations and high pollution levels (OPEC 2014).

Furthermore, it has to be expected that the sulfur level in on-road and off-road gasoline and diesel requirements will become stricter in the foreseeable future towards ultra-low sulfur fuels, approaching zero sulfur emissions from burned fuels. Therefore, the efficiency of the desulfurization technologies becomes a key point (Alves *et al.* 2015).

1.4. Hydrodesulfurization

Hydrodesulfurization (HDS) is the most commonly used industrial process for the removal of sulfur compounds from crude oil and refined petroleum products. As shown in **Figure 4**, this process consists in the use of hydrogen to convert organic sulfur into hydrogen sulfide (H_2S), which is easily removed. It combines high pressure and temperature (150 – 3000 psi; 250 – 455°C) in contact with crude oil, and uses $CoMo/Al_2O_3$ or $NiMo/Al_2O_3$ as metal catalysts (Whitehurst *et al.* 1998; Shafi & Hutchings 2000; Javadli & Klerk 2012).
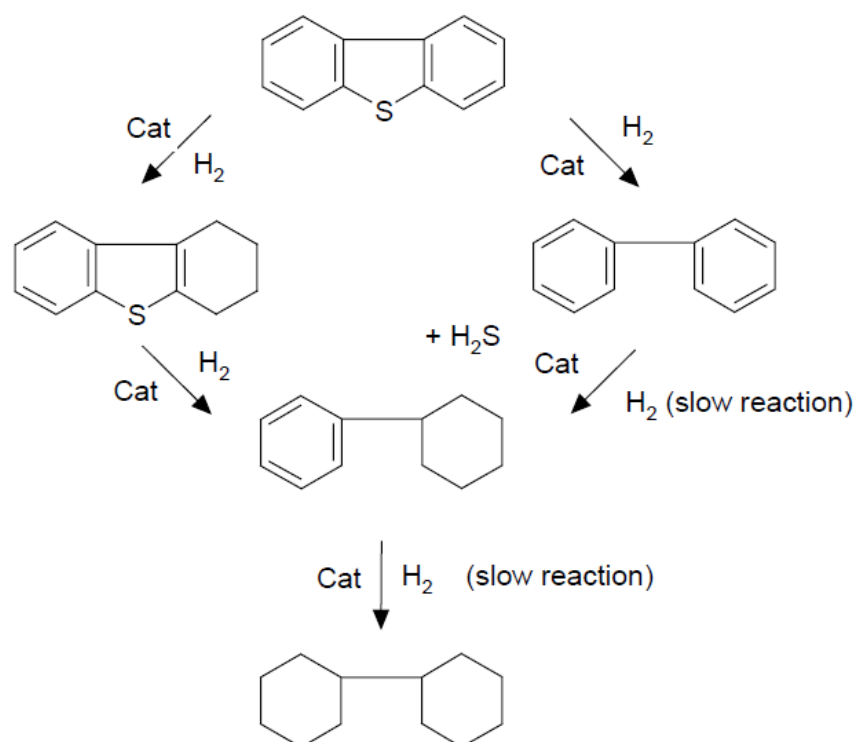
Figure 4 – Proposed reaction mechanisms for DBT hydrodesulfurization (Cat = catalyst). (Adapted from Marcelis, 2002).

Although HDS is the most widespread physico-chemical method for sulfur removal from crude oil, sometimes it is still not enough to reach the increasingly stringent legal limits established. The presence of polycyclic aromatic compounds, such as DBT and its derivatives (abundant in heavy crude oils), leads to a lower efficiency of sulfur removal. The complex structural characteristics of these molecules protects the sulfur atom from the hydrogen attacks, maintaining its structure intact.

Thus, to achieve lower sulfur concentrations, it is necessary to increase the intensity of the physico-chemical treatment implementing deep HDS.

Deep HDS is a process that uses higher temperatures and pressures and longer residence times with more sophisticated catalysts (Klein 1999), so it has several drawbacks, which are direct consequences of the extreme conditions applied towards desulfurization. Deep HDS makes the process of removing the organic sulfur highly expensive and substantially increases its carbon footprint (Singh *et al.* 2012). It reduces the quality of the final product, as the higher temperatures and pressures used allow the conversion of other chemical components present in the fuel, therefore decreasing the octane number (Folsom *et al.* 1999; Reichmuth *et al.* 2000; Javadli & Klerk 2012). The lifespan of the used catalysts is also shortened by the poisoning effect of the generated H_2S (the heavier the feedstock, the faster the catalyst deactivates) and the deactivated catalysts constitute a hazardous type of waste which poses several disposal problems (Vogelaar 2003; Bhatia & Sharma 2012). Deep HDS is largely affected by the low-reactivity components in the reaction mixture, such as organic hetero-compounds and polyaromatic hydrocarbons (Monticello 1996; Folsom *et al.* 1999; Konishi *et al.* 2000; Egorova 2003).

Since the production of ultra-low sulfur automotive fuels has gained enormous interest in the scientific community worldwide, other desulfurization technologies, much more efficient and less expensive in removing HDS recalcitrant organic sulfur compounds, are being used in test scale and commercial scale projects. These include oxidative desulfurization (ODS), extractive desulfurization, adsorptive desulfurization and biodesulfurization (Babich & Moulijn 2003; Adegunlola *et al.* 2012; Muzic & Sertic-Bionda 2013). Among them, ODS combined with extraction (OEDS) or adsorption is considered to be one of the most promising processes to remove refractory sulfur compounds from diesel fuel (Sampanthar *et al.* 2006; Jiang *et al.* 2011). However, for heavy oil desulfurization, few of these processes are viable and/or as effective as HDS. The approach with the best chance of leading to a breakthrough in desulfurization of heavy oil is autoxidation followed by thermal decomposition of the oxidized heavy oil. There is also possibility for synergistically employing autoxidation in combination with biodesulfurization and hydrodesulfurization (Javadli & Klerk 2012). Therefore, the study for a more efficient and profitable way to remove sulfur from crude oils is still mandatory.

1.5. Biodesulfurization

Nowadays, biotechnology has replaced several chemical processes, since biocatalysts can function at mild conditions and more efficiently. This brings many advantages such as the reduction of energy costs, lower emissions and minimal generation of undesirable by-products (Borgne & Quintero 2003; Singh *et al.* 2012). It is known that oil refining processes are mainly physico-chemical, so biotechnological approaches to the problems presented tend to be very well received. Several microorganisms and their enzymes have already been studied on both bench and pilot scale for

application on several parts of the refining process, such as biodenitrogenation, biodemetallation, biodemulsification and microbial enhanced oil recovery (MEOR), biological control of reservoir souring and transformation of heavy crude to light crude and biodesulfurization (Singh *et al.* 2012).

Biodesulfurization (BDS) is a biotechnological process corresponding to HDS, since it removes sulfur from complex compounds. In the last decade, BDS has drawn wide attention because of its green processing of fossil fuels. It takes place at low temperature and pressure, through the use of microorganisms capable of metabolizing sulfur compounds, and, in the last 20 years, has been extensively studied as an alternative to HDS (Soleimani *et al.* 2007; Alves & Paixão 2011). BDS is able to remove the most recalcitrant sulfur compounds (for example, DBT), without the use of hydrogen and without the formation of H_2S. In fact, the removed sulfur is used by the microorganism in its own metabolic pathways and it is eventually converted into biomass, turning the process much more eco-friendly (Caro *et al.* 2007; Mohebali & Ball 2008). The maintenance of a desulfurizing microorganism is also cheaper than the purchase or development of new chemical catalysts. When compared to HDS, this process requires approximately two times less capital and 15% less operating costs (Kaufman *et al.* 1998; Mohebali & Ball 2008).

There are very few reports on BDS process designs and cost analysis (Gupta *et al.* 2005). Recently, Alves *et al.* (2015) reported a study where two BDS process designs were analyzed in terms of energy consumption, greenhouse gas emissions (GHG) and costs. This study pointed out for the application of the BDS downstream HDS as the best cost-effective conceptual design to apply into an oil refinery. Since it is able of desulfurize HDS recalcitrant compounds selectively, BDS integration may lead to the accomplishment of the stringent European limit of <10 ppm for S-content on fuels, which otherwise may imply the necessity of more severe conditions within HDS units. Deep HDS is a very costly option and is not environmentaly friendly because implies higher GHG emissions and substantially increases the carbon footprint.

1.5.1. Dibenzothiophene and biodesulfurization pathways

The model compound most used in BDS studies is the dibenzothiophene (DBT) (**Figure 5**). It consists of two benzene rings fused to a central thiophene ring which encases the sulfur molecule. DBT is an abundant and very recalcitrant thiophene derivative that resists even the action of deep HDS and it is easily manipulated, since it is not mutagenic or hazardous to human health, hence its choice for most studies (Alves 2007).

DBT desulfurization can be performed both by anaerobic and aerobic microorganisms. Through the anaerobic pathway microorganisms convert DBT into biphenyl with release of H_2S as a side-product. The anaerobic pathway was firstly described by Kim *et al.* (1995), who reported a significant conversion of model compounds by a concentrated cell suspension of *Desulfovibrio desulfuricans* M6, a sulfate reducing bacterium with high hydrogenase activity. Anaerobic biodesulfurization might

be an alternative to conventional refineries, since there is no production of colored or gum forming side products (McFarland 1999). However, growth under anaerobic conditions proceeds slowly, even more when organic molecules are involved, and maintaining an anaerobic environment might be somewhat difficult in large scale.
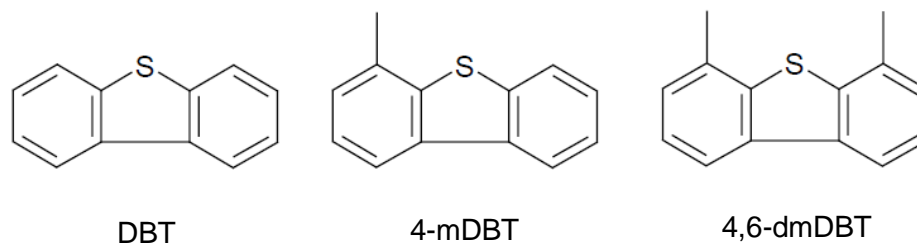
Figure 5 – Structural formulas of refractory methylated DBT. (Adapted from Marcelis 2002).

The aerobic pathway for DBT desulfurization was firstly described by Kodama *et al.* (1973) and became known as the Kodama pathway. This pathway consists in the dihydroxylation of the peripheral aromatic ring of the DBT molecule, followed by its cleavage into 3-hydroxy-2-formyl-benzothiophene. This compound is water-soluble and maintains the sulfur atom, but has lower carbon content compared to DBT.

About 20 years later the Van Afferden pathway was described. This is a carbon consuming pathway present in the bacteria *Brevibacterium* sp. DO, which can use DBT as a sole carbon and sulfur source. During DBT mineralization there is an oxidation into DBT sulfone, then an aromatic dioxygenase causes the rupture of the thiophenic ring to form 2,3-dihydroxybiphenyl 2'-sulfinate DBT. Finally, a second action of the dioxygenase opens the 2,3-dihyroxybenzene nucleus, resulting in sulfite. This compound is then oxidized to sulfate and benzoate, being the latter mineralized into CO_2 and H_2O (Afferden *et al.* 1993).

A third aerobic pathway was described by Kilbane (1989) from an isolated *Rhodococcus erythropolis* strain exhibiting a sulfur specific pathway, able to carry out a stepwise selective oxidation of the hetero sulfur atom while the carbon skeleton is not metabolized. *Rhodococcus erythropolis* strain IGTS8 appeared to be capable of using a wide range of organic sulfur compounds as the sole source of sulfur, for example thiophenes, sufides, disulfides, mercaptans, sulfoxides and sulfones (Kayser *et al.* 1993). In particular, sulfur is removed from DBT originating the end-product 2-hydroxybiphenyl (2-HBP) (Gallagher *et al.* 1993). The sulfur specific metabolic pathway for DBT desulfurization involves four enzymatic steps and is designated the 4S-pathway (**Figure 6**).

Rhodococcus erythropolis strain IGTS8 desulfurizes DBT using three enzymes DszA, DszB and DszC, which are located at the plasmid-encoded *dsz* operon. Denome *et al.* (1993; 1994) and Piddington *et al.* (1995) provided insight in the sequence of reactions by identifying and cloning the responsible genes for desulfurization: *dsz*A, *dsz*B and *dsz*C. Oldfield *et al.* (1997) succeeded in the

conclusive elucidation of the 4S-pathway by analyzing its intermediates and products. A mono-oxygenase (DszC) catalyses the stepwise S-oxidation of DBT, first to dibenzothiophene 5-oxide (DBTO) and then to dibenzothiophene 5,5-dioxide ($DBTO_2$). The second mono-oxygenase (DszA) catalyses the conversion of $DBTO_2$ to 2-(2'-hydroxyphenyl) benzene sulfinate (HBPS). The last step is catalyzed by a sulfinase (DszB) and yields 2-HBP and sulfite as the end-products (Marcelis 2002). There is also a fourth enzyme, FMN-reductase (DszD), which regulates the activity of the mono-oxygenases by adjusting the levels of the reduced flavin (Nomura *et al.* 2005).
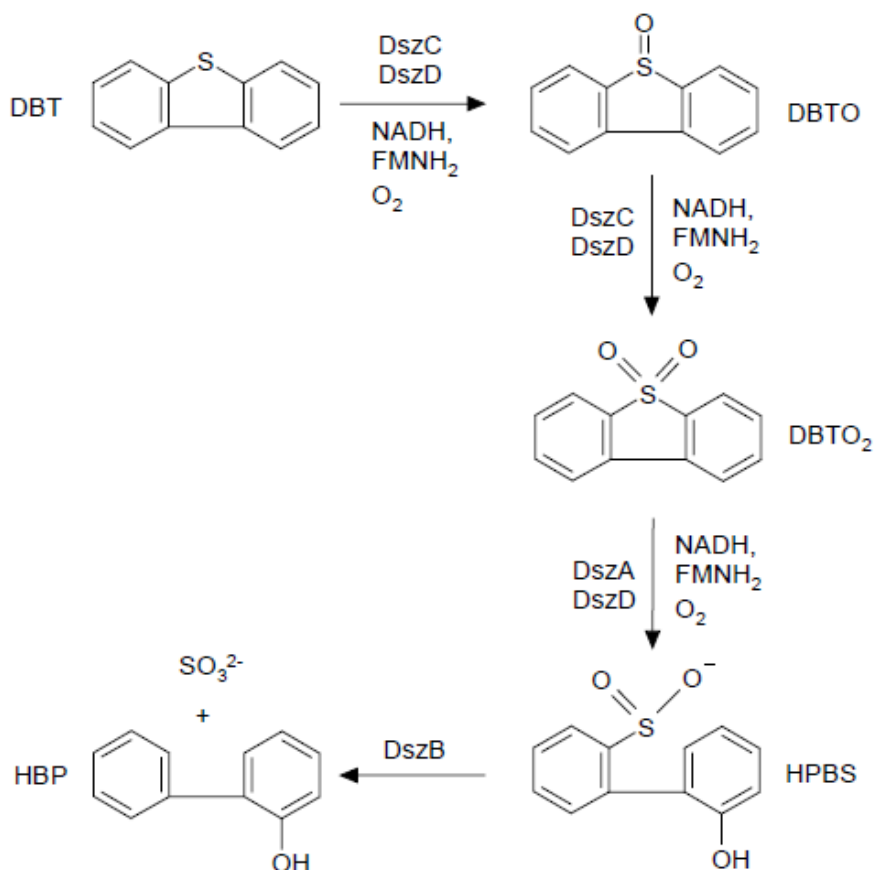
Figure 6 – *4S-pathway as performed by* R. erythropolis *IGTS8. (Adapted from Marcelis, 2002).*

Unlike the Kodama and Van Aferdeen pathways, the 4S-pathway enables the removal of sulfur without compromising the carbon skeleton of the molecule. Therefore the calorific power is not affected, making it the best pathway for the desulfurization of crude oils. Another advantage of the 4S-pathway is that it can proceed even when cells are not growing. However, it is necessary to provide the essential co-factors to sustain the redox reactions (Martinez *et al.* 2014).

In the last decades, several studies have been conducted using bacterial genera capable of using the 4S-pathway for BDS, which include *Arthrobacter*, *Agrobacterium*, *Brevibacterium*, *Klebsiella*, *Mycobacterium*, *Nocardia*, *Paenibacillus*, *Pseudomonas*, *Xanthomonas*, *Rhodococcus* and *Gordonia*,

being these last two the most promising (Clark & Kirk 1994; Tanaka *et al.* 2002; Alves *et al.* 2008; Alves & Paixão 2011; Silva 2012; Alves & Paixão 2014).

1.6. *Gordonia alkanivorans* strain 1B

The genus *Gordonia* is very diverse, and was firstly proposed as the genus *Gordona* by Tsukamura (1971), being then renamed as *Gordonia* by Stackerbrandt *et al.* (1988). This genus comprises gram positive, aerobic, non-sporulating bacteria and is part of the family *Gordoniaceae*, order *Actinomycetales*, sub-order *Corynebacterineae*. In fact, *Gordonia* is closely related to genus as *Mycobacterium*, *Nocardia* and *Rhodococcus*, all part of the same sub-order (Goodfellow & Maldonado 2006). Research about this genus has been increasing since 1998, and 36 different species have already been described (NCBI 2015). Some of them, such as *G. aichiensis*, *G. araii*, *G. bronchialis*, *G. effuse* and *G. sputi* have been isolated from imuno-compromised clinical patients. However they are not considered harmful for healthy individuals (Drzyzga 2012). The majority can be found in several aquatic and terrestrial habitats, meaning that members of the genus *Gordonia* are widely distributed in the environment as is generally assumed for other mycolic acid-containing actinomycetes such as *Rhodococcus* and *Mycobacterium* (Drzyzga *et al.* 2011).

Many species from the genus *Gordonia* are attractive for biotechnological uses due to their ability to degrade several environmental pollutants as polycyclic aromatic hydrocarbons, alkylpyridines, phthalates, xenobiotics or slowly biodegradable natural polymers. They are also attractive due to their ability to transform or synthetize organic compounds, such as steroid transformation and carotenoid production (Harner *et al.* 2011; Grace Liu *et al.* 2011).

The bacteria *G. alkanivorans* strain 1B, the microorganism used in the present study, was isolated by Alves *et al.* (2005) from oil contaminated ground samples from Parque das Nações (Lisbon, Portugal). It is an aerobic, gram-positive, catalase-positive, oxidase-negative and pink/orange-pigmented bacterium. Cells were shown to be short branched hyphae, which disintegrated to rods and coccus-like elements when visualized by phase contrast microscopy. They are non-motile cells generally occurring in groups (Alves *et al.* 2005).

1.6.1. Desulfurization ability

The strain 1B was first described as a desulfurizing bacteria in 2005, being able to convert DBT to 2-HBP at a rate of 2.58 μM.h^{-1}, corresponding to a maximum specific desulfurization rate ($q_{2\text{-HBP}}$) of 1.03 μmol(2-HBP).g^{-1}(Dry Cell Weight or DCW).h^{-1}, using glucose as the single carbon source (Alves *et al.* 2005). Similar results were reported for another strain of the same species, *Gordonia alkanivorans* strain RIPI90A, which obtained a specific desulfurization rate of 1.40 μmol.g^{-1}(DCW).h^{-1} (Mohebali & Ball 2008).

Alves *et al.* (2007), studying the *G. alkanivorans* strain 1B desulfurization genes, proved the existence of the *dsz* operon in the genome of this strain. These genes were compared with other sequences of relevant desulfurizing microorganisms and showed high similarity with those from *R. erythropolis* IGTS8 (88% for *dsz*A, 88% for *dsz*B and 90% for *dsz*C). The *dsz*AB genes were later cloned and expressed in *E. coli*, which became able of using dibenzothiophene sulfone (DBTS) as a sulfur source and desulfurize it 4.5 times faster, without the substrate inhibition seen in *G.alkanivorans* strain 1B (Alves *et al.* 2006).

1.6.2. Fructophilic behavior and its influence on desulfurization ability

Usually fructophilic pathways are associated to yeasts, and the only known prokaryotes presenting this behavior were lactic-acid bacteria (Leandro *et al.* 2011; Neveling *et al.* 2012). However, Alves & Paixão (2014b), were able to describe a fructophilic behavior in *G. alkanivorans* strain 1B. When cultivated with fructose as carbon source it was able to achieve an optical density 5-fold higher than with glucose after only 5 days, increasing its growth rate from 0.025 h^{-1} to 0.091 h^{-1}. The greater number of functional cells produced in fructose conducted to a more effective BDS process by strain 1B, as it was attained a $q_{2\text{-HBP}}$ about 74% higher than in glucose grown cultures (2.10 $\mu mol.g^{-1}(DCW).h^{-1}$ in fructose *versus* 1.20 $\mu mol.g^{-1}(DCW).h^{-1}$ in glucose). Moreover, this significant BDS enhancement can be better observed in terms of the overall 2-HBP production rate, which increased over 5-fold, from 1.8 $\mu M.h^{-1}$ (in glucose) to 9.29 $\mu M.h^{-1}$ (in fructose).

This discovery opened a new line of research, where new alternative carbon sources could be harnessed to make the biodesulfurization process industrially viable and profitable. In this ambit, the potential of several sustainable fructose-rich materials (agro-industrial materials), such as sugar beet molasses, carob pulp, strawberry tree fruit and Jerusalem artichoke tubers, were evaluated as alternative carbon sources towards cost-effective DBT desulfurization by strain 1B (Silva 2012; Silva *et al.* 2012; Silva *et al.* 2015; Alves & Paixão 2014a). All these tested alternative carbon sources yielded better desulfurization results when compared with glucose rich carbon sources, however the greater results obtained using Jerusalem artichoke (2-HBP production rate = 28.2 $\mu M.h^{-1}$ and $q_{2\text{-HBP}} = 5.06\ \mu mol.g^{-1}(DCW).h^{-1}$) highlighted its potential as the best alternative carbon source towards the upscale of BDS by strain 1B (Silva 2012). Therefore, in this work Jerusalem artichoke juice was chosen as the preferred cost-effective alternative carbon source to be added towards culture medium optimization.

1.7. Jerusalem artichoke as a sustainable carbon source for BDS

Heliantus tuberosus (**Figure 7**) or Jerusalem artichoke (common designation) is a species of sunflower originated from eastern North America, from Maine west to Dakota and southwards to northern Florida and Texas. It is now widely cultivated worldwide, including countries such as France,

Italy, Portugal, Germany, East European countries, China and also some tropical countries in the cooler highlands (India, Indonesia, Malaysia, Kenya, Zaire and Nigeria) (Lim 2015).

Although it is a temperate species, the Jerusalem artichoke has good tolerance to frost, draught and other adverse conditions, as well as resistance to pests and diseases (Slimestad *et al.* 2010). It is moderately resistant to salinity, being able to grow in salt-affected land with 25 to 50% seawater irrigation and at different soil pHs (Kosaric *et al.* 1984; Zhao *et al.* 2008). These properties make it a good type of culture for the use in biotechnological processes, since it is not limited by specific growth conditions.

Figure 7 – <u>Heliantus tuberosus</u> flower and tubers. Photographs by David G. Smith (flowers) and Robert White/Corbis (tubers)

The Jerusalem artichoke tubers are a rich source of inulin (**Figure 8**), a linear fructan in which a variable number of fructose units are linked by β (2 →1) bonds. Glucose typically ends the inulin chain through a α (1 → 2) bond. Inulin is soluble in water and, after hydrolytic breakdown (acidic or enzymatic hydrolysis), the reducing sugars fructose and glucose can be easily released (Silva *et al.* 2015). The Jerusalem artichoke is an excellent crop for inulin production, and the USA, Russia and some European countries use it in several industrial processes (Ziyan & Pekyardimci 2003).
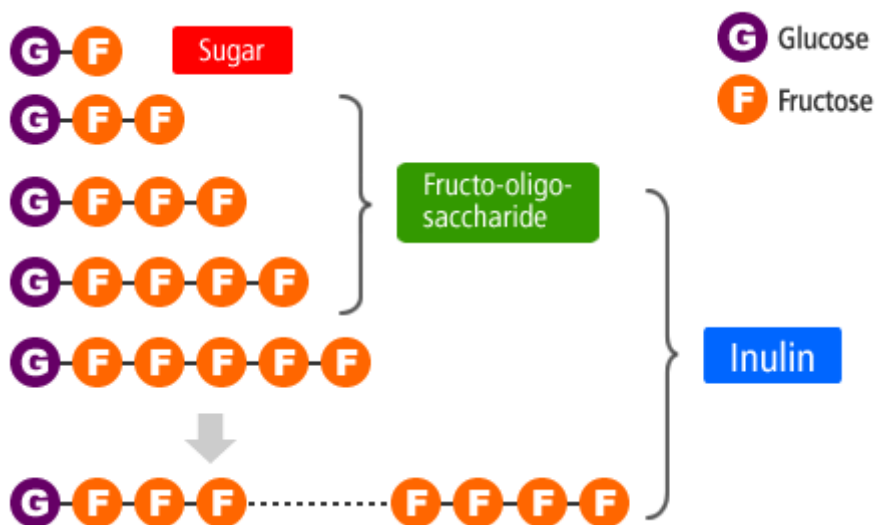

Figure 8 – Chemical structure of inulin. (Adapted from http://www.fnsugar.co.jp/eng/inulin.html)

This plant already has many industrial applications. The herbage was found to be a good source of a high-quality protein isolate, rich in lysine, which may provide good ruminant feed (Rawate & Hill 1985). The tubers are an important fructose source that can be used as sweetener in the food industry (Pilnik & Vervelde 1976). Anaerobic digestion experiments showed that fresh and ensiled above-ground parts of the plant could produce up to 680 l of biogas per kg of organic material (Gunnarson *et al.* 1985).

Jerusalem artichoke also has a great deal of unused potential as feedstock for the production of bioethanol, using inulin-adapted strains or inulinase-producing strains of yeasts for fermentation. Its tubers have been reported to have one of the highest carbohydrate yields of known agricultural crops, ranging between 900 and 2400 kg sugars per acre per year, which is equivalent to about 1000 – 2400 kg ethanol per acre per year assuming an ethanol yield of 80% of the maximum theoretical yield *(Margaritis et al.* 1981). In fact, Jerusalem artichoke was used as raw material for the production of motor fuel alcohol during the Second World War and has been sporadically used for this purpose since then (Chekroun *et al.* 1996). It is also feasible to produce biodiesel from Jerusalem artichoke tuber, using the heterotrophic microalga *Chlorella protothecoides* (Cheng *et al.* 2009).

Several Jerusalem artichoke genotypes were found to have promising soil phytoremediation activity in removal of heavy metal pollutants like cadmium from contaminated soil (Long *et al.* 2013). In pot experiment studies, Jerusalem artichoke was able to remove heavy metals as Cd, Pd, Ni, Cu and Zn from heavy metal-contaminated soils and accumulating them in the plant (Jasiewicz & Antonkiewicz 2002). Ma *et al*. (2011) reported that it has been widely cultivated in Shanxi, Heilongjiang, Shandong and Jiangsu provinces to improve salt-alkaline soils, oil-polluted soils and coal-mining soils. It was also reported as antifungal and weed control. The ethyl acetate extracted from the leaves exerted antifungal activity, with inhibitory rates of 77.9, 100 and 100% against the plant pathogens *Rhizoctonia solani*, *Alternaria solani* and *Botrytis cinerea*, respectively (Liu *et al.* 2007; Tesio *et al.* 2011).

These factors make the Jerusalem artichoke a sustainable carbon source towards bioprocesses (e.g. bioethanol production; BDS). Since inulin is rich in fructose, it is an ideal source for the growth of *G. alkanivorans* strain 1B. Silva *et al.* (2015) described the optimized desulfurization process by strain 1B using Jerusalem artichoke juice (JAJ) as carbon source. Prior to its utilization, JAJ had to be hydrolyzed and its sulfates removed by precipitation with $BaCl_2$. This treatment enabled desulfurization of DBT by *G. alkanivorans* strain 1B yielding a q_{2-HBP} of 5.06 $\mu mol.g^{-1}(DCW).h^{-1}$ which is 4- to 7-fold higher than those reported in prior BDS studies with other alternative carbon sources and very similar to what the author obtained with fructose as pure carbon source (Silva *et al.* 2012; Silva *et al.* 2015).

1.8. Challenges towards biodesulfurization industrial scale-up

Since BDS is more efficient and less expensive than HDS in removing sulfur from refractory heterocyclic compounds present in crude oil, it could be used in oil refinery as a complement to achieve ultra-low sulfur diesel (Alves *et al.* 2015). BDS can be an alternative/complement to desulfurize heavy oils, like shale oils, which have high thiophene concentration. However, to apply BDS into an industrial scale it is mandatory to overcome several limitations and problems.

BDS shows a low conversion rate, which may be caused by feedback inhibition of the enzyme activities by the accumulation of the end product of the 4S-pathway (2-HBP), which is extremely toxic to most microorganisms (Alves & Paixão 2011). The 4S-pathway is also inhibited in the presence of other sulfur compounds in the culture medium, and even small concentrations of sulfate or other easy access sulfur sources can inhibit BDS (Mohebali & Ball 2008; Silva *et al.* 2012). To add up, bacteria have very low sulfur requirements. In *Rhodococcus* sp., the cells were found to require 0.1 mM of sulfur for normal growth (Reichmuth *et al.* 2000). Only 1% of bacterial dry weight is sulfur, which implies a very low need in relation to this element (Stoner *et al.* 1990). Therefore, it is important to use feedstocks containing low residual concentration or even null content of sulfur (Silva 2012).

Moreover, there is a limitation associated with the costs of the culture medium. This is a problem shared with many other biotechnological processes, representing 30 to 40% of the total value, namely the carbon source, which makes the process more expensive (Alves *et al.* 2015). At present, there is no economically suitable method for large-scale preparation of biocatalysts (Tao *et al.* 2006). Since it is necessary to reduce production costs, it is important to search for cheap and widely available raw materials. The utilization of alternative carbon sources derived from agro-industrial by-products, wastes or plants that can be grown on marginal land may represent a sustainable strategy for biotechnological processes (Alves *et al.* 2008; Alves & Paixão 2014a; Silva *et al.* 2013; Silva *et al.* 2015). Many carbon sources were already tested in the search for a suitable alternative for the production of biocatalysts able to perform BDS. The first reported utilization of raw materials as carbon sources for this process was by Alves *et al.* (2008), using recycled paper sludge which is a very abundant type of waste resultant of the pulp and paper industry. Recycled paper sludge is very rich in cellulose, xylan and lignin which can be hydrolyzed into glucose, xylose and cellobiose, making it very interesting as a carbon source. After this work, other alternative carbon sources have been tested, with different degrees of success, such as glycerol (Abo-State *et al.* 2014; Tang & Hong 2014), carob pulp liquor (Silva *et al.* 2012), Jerusalem artichoke juice (Paixão *et al.* 2013; Silva *et al.* 2015) and sugar beet molasses (Alves & Paixão 2014a).

Until recently, BDS studies have been focused mainly on model compounds, and this fact also limits the ability to demonstrate the commercial potential of this biotechnology (Grossman *et al.* 2001; Alves 2007). Oils are complex substrates, therefore, it is necessary to take into account their associated

problems such as the toxicity of their constituent compounds and the existence of steric hindrance associated with the molecular structure of these compounds that might inhibit the activity of microbial enzymatic systems (Alves 2007). However, recent studies with petroleum show sulfur removal up to 90%, using different microorganisms and on different distillates, being achieved an ultra-low sulfur diesel with 14 ppm of S-content (sulfur content) (Bhatia & Sharma 2012; Kilbane 2006; Nuhu 2013).

For the industrial production of the biocatalyst, the use of a continuous flow stirred tank reactor (CFSTR), instead of a fed batch system, can bring many advantages (Pacheco 1999). In this system cells are always at their maximum growth rate and therefore, all the metabolic pathways are at their highest function rate. This system also has the advantage of not accumulating end products which can inhibit several pathways, including desulfurization, being an asset for metabolic related bioprocesses (Schilling *et al.* 2002).

However, the best approach to efficiently perform BDS is still not consensual. Some authors affirm that the best approach is to grow the biocatalysts in the same reactor as the BDS is to be performed (Schilling *et al.* 2002). Others state that to efficiently perform BDS it is needed a biphasic system where the biocatalyst and the crude oil are only in contact in a thin layer, where BDS occurs. This system is proposed by (Yang *et al.* 2007), and is based in the use of a biphasic continuous stirred tank reactor, since the partition of organic and inorganic phases brings many advantages in the down-stream processing. Another interesting alternative is the immobilization of the biocatalysts, which allows an easier recovery and re-use of cells, increased stability to different reaction conditions and components and lower biocatalyst contamination of final product (Zhang *et al.* 2010).

In overall, there are several problems related to large scale operation, such as the difficulty in the control of various parameters that regulate biocatalyst activity and stability, the oil/water volume ratio, the logistics of sanitary handling, shipment, storage and use of effective microorganisms within the production field or refinery environment (Javadli & Klerk 2012). There are also some problems associated with the biphasic system used for BDS. The cells, when mixed with water and oil, produce a sort of surfactant, which emulsifies the oil and makes the separation from water and cell recovery highly difficult (Alves 2007; Naito *et al.* 2001).

When successfully implemented, BDS technology could result in less environmental issues, with a CO_2 emission reduction of 70 to 80%, smaller residue production and reduced energy consumption, which, in turn, is translated in reduced capital (two thirds of HDS) and operational costs (Vazquez-Duhalt *et al.* 2002; Alves *et al.* 2015). In addition, the BDS process may benefit from the simultaneous production of high-added value microbial by-products (such as biosurfactants and pigments), which can be further valorized through other industrial processes (De Miguel *et al.* 2000; De Miguel *et al.* 2001; Veiga-Crespo *et al.* 2012; Bandyopadhyay & Chowdhury 2014). The final product of the 4S-pathway (2-HBP) is a hydrotrope that can act as a surfactant which could be

recovered from BDS effluents (Mohebali *et al.* 2007). On the other hand, Alves *et al.* (2005) described *Gordonia alkanivorans* strain 1B as a pink/orange pigmented bacteria. Orange color is normally associated with carotenoids as β-carotene, so this aspect can be exploited taking into account a further industrial scale-up of the BDS process using this bacterium (Veiga-Crespo *et al.* 2012).

1.9. Scope of the thesis

Due to the strict European regulations regarding the legal limits on sulfur concentration on fossil fuels and their derivatives it is imperative the development of cost-effective technologies to remove sulfur. Therefore, the optimization studies of BDS as an eco-friendly technology complementary to HDS towards an integrated industrial production of ultra-low sulfur fuels (gasoline/diesel) in a petroleum refinery is an important focus of research.

In this context, the main goal of this study consisted on the component minimization of the culture medium for the maximum production of efficient desulfurizing biocatalysts (*G. alkanivorans* active cells) taking into account the lowest operation costs. In fact, the utilization of desulfurizing microorganisms that can grow in low nutrient culture media without vitamins and/or other growth promoters (*e.g.* yeast extract, peptone, triptone, etc) is an advantage for BDS upscale since it may reduce the biocatalyst production costs significantly. Hence, several objectives were developed:

- Minimization of the sulfur free mineral (SFM) media, usually used to grow *Gordonia alkanivorans* strain 1B, in shake-flask assays;
- Optimization of SFM medium minimization in chemostat assays;
- Optimization of biocatalysts production (resting cells of *G. alkanivorans* strain 1B) in chemostat using the minimized medium with the best culture conditions and JAJ as sustainable carbon source;
- Evaluation of the desulfurization ability of a model oil by *G. alkanivorans* strain 1B grown in the chemostat optimized conditions.

2. Materials and methods

2.1. Chemicals and reagents

Dibenzothiophene (DBT 99%) was obtained from Acros Organics (Geel, Belgium), 2-hydroxybiphenyl (2-HBP) was from Sigma (Missouri, USA), dimethylformamide (DMF) was from Riedelde Haën (Hannover, Germany), 4-methyl-DBT (96%) was from Aldrich Chem. Co. (Missouri, USA), $BaCl_2.2H_2O$ (>99%) and sodium sulfate anhydrous (>99%) were obtained from Merck (New Jersey, USA). For all BDS assays, a stock solution of 150 mM DBT, dissolved in DMF, was prepared. The 5,6-

carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) were acquired from Invitrogen (Massachusetts, USA). All other reagents were of the highest grade commercially available.

2.2. Jerusalem artichoke juice

Jerusalem artichoke tubers were cultivated in a depleted soil in Oleiros, Portugal and processed as described in Silva *et al.* (2015) towards the production of Jerusalem artichoke juice (JAJ). The JAJ was hydrolyzed at pH 2 and 55°C for 48 hours to convert inulin into fructose and glucose and then filtered (0.20 μm membranes). For total sulfate precipitation, sterile $BaCl_2$ at 0.5% (w.v^{-1}) was added to the filter sterilized juice adjusted to pH 8.73 (Silva *et al.* 2015). This JAJ was shaken vigorously, incubated at 30°C for 36 hours and finally filtered to remove all $BaCl_2$ (0.45 μm membranes) and again filter sterilized (0.20 μm membranes) for storage effects. This JAJ precipitated with $BaCl_2$ for sulfate removal (JAJ_p) was used in minimized medium in shake-flask assays.

A second process of JAJ preparation was tested, based in inulin precipitation. Untreated juice was frozen at -20°C and then defrosted at 4°C, in order to precipitate the inulin. When two phases were formed the upper layer was removed. The bottom layer containing pulp and precipitated inulin was then hydrolyzed in the same conditions described above to obtain a JAJ with the correspondent monosaccharides. In this process, no sulfate precipitation was performed. This JAJ concentrate (JAJ_c) was used in minimized medium in chemostat/bioreactor assay, as an alternative carbon source.

2.3. Microorganism and culture media

The microorganism used in this study was the bacterium *Gordonia alkanivorans* strain 1B, isolated in our laboratory (Alves *et al.* 2005). The basal salts medium used for cultivation and maintenance of this microorganism was a sulfur-free mineral (SFM) culture medium containing 1.22 g.l^{-1} NH_4Cl, 2.55 g.l^{-1} KH_2PO_4, 2.55 g.l^{-1} $Na_2HPO_4.2H_2O$ and 0.17 g.l^{-1} $MgCl_2.6H_2O$. This medium was supplemented with 0.50 ml.l^{-1} of a sulfur-free trace elements solution (TES) (Alves & Paixão 2014b) and its final pH was adjusted to 7.5, before being autoclaved at 121°C, 1 atm for 15 minutes. Pure carbon sources such as glucose and fructose were dissolved in Millipore water in 50% (w.v^{-1}) concentrated solutions. Pure carbon sources and Jerusalem artichoke juice (JAJ) were filter sterilized with a 0.20 μm pore sterile filter before being added to the medium in aseptic conditions, to an initial concentration of ~10 g.l^{-1} total reducing sugars.

Usually, prior to each assay, a bacterial inoculum was prepared by growing the strain 1B with fructose (10 g.l^{-1}) as the single carbon source and 250 μM DBT as single sulfur source. This fermentation was performed in a volume of 150 ml, in a 500 ml shake-flask, at 30°C and 150 rotations per minute (rpm) in a horizontal incubator (Unitron CH-4103, Infors AG, Bottingen, Switzerland) for 72 hours.

2.4. Culture medium minimization: shake-flask assays

Culture medium minimization assays consisted in BDS assays in which different formulations of the SFM culture medium, above described, were tested with the aim of designing a minimal culture medium that did not limit growth or desulfurization by strain 1B. The key culture medium components minimized were: nitrogen (NH_4Cl), magnesium ($MgCl_2.6H_2O$) and TES.

BDS assays were performed in 500 ml shake-flasks with 150 ml of total volume, by growing a bacterial inoculum of 2% (v.v^{-1}), prepared previously as above described, in different formulations from SFM medium, varying the nitrogen/magnesium/TES concentrations (ranging from 0% to 75% of the SFM original amounts) but maintaining the carbon and sulfur sources concentration (10 g.l^{-1} total reducing sugars; 250 µM of DBT). The details of the tested medium formulations, #1 to #15, are resumed in the **Table 2**, being #1 the original SFM medium (control assay). All BDS assays were carried out in triplicates.

Table 2 – *Different formulations of the SFM media, with the amounts of the components NH4Cl, MgCl2.6H2O and TES, tested in shake-flask assays towards the design of the optimal minimal culture medium to produce effective desulfurizing biocatalysts.*

Set assays	Medium formulations (F#)	SFM culture medium composition			Carbon source (10 g.l^{-1})[c]	Sulfur source (250 µM)
		Components minimized				
		NH$_4$Cl (g.l^{-1})	MgCl$_2$.6H$_2$O (g.l^{-1})	TES (ml.l^{-1})		
Control ([a]SFM)	#1 #1'	1.22	0.17	0.50	8 Fru + 2 Glu JAJ$_p$	DBT
N Minimization	#2	0.92 **(75%)**	0.17	0.50	8 Fru + 2 Glu	DBT
	#3	0.61 **(50%)**				
	#4	0.31 **(25%)**				
Mg Minimization	#5	1.22	0.13 **(75%)**	0.50		
	#6		0.09 **(50%)**			
	#7		0.04 **(25%)**			
TES Minimization	#8	1.22	0.17	0.38 **(75%)**		
	#9			0.25 **(50%)**		
	#10			0.13 **(25%)**		
	#11			0.03 **(5%)**		
	#12			0.01 **(2.5%)**		
	#13			0 **(0%)**		
Minimized Medium ([b]SFMM)	#14 #15	1.04 **(85%)**	0.05 **(25%)**	0.13 **(25%)**	8 Fru + 2 Glu JAJ$_p$	DBT

[a]SFM – Sulfur-free mineral medium (default medium for shake-flask assays)

[b]SFMM – SFM minimum media for shake-flask

[c]10 g.l^{-1} total sugars; 8 Fru: fructose 8 g.l^{-1}; 2 Glu: glucose 2 g.l^{-1}; JAJ$_p$: Sulfate precipitated Jerusalem artichoke juice

2.5. Minimum culture medium: optimization in chemostat

The continuous culture assays were performed on a bench-top bioreactor BioFlo IIc (1 l vase) with 390 ml working volume (New Brunswick Scientific, Edison, New Jersey, USA). NH_4Cl screening assays, were performed in a tailor-made bioreactor system coupling a GLS 80® stirred reactor (500 ml Duran® GLS80 bottle + screw cap connection system + stirrer shaft from Duran Group GmbH, Germany) with 260 ml working volume. The working volume was maintained constant by a surface dipped leveling tube linked to a peristaltic pump with adjustable speed while the influx of culture medium was kept constant by a second peristaltic pump. In the continuous culture assays, independently of the reactor used, the temperature was kept at 30°C, the pH was controlled by the addition of 1 M NaOH solution on demand to maintain pH at 7.5, and agitation was kept at 300 rpm. The aeration rate was 2 vvm.

The starting culture medium used for this set of reactor minimization assays was an adaptation of the SFM medium (designated as SFM_R medium), which was previously optimized in our lab for minimal phosphate source. This SFM_R medium contained 1.22 g.l^{-1} NH_4Cl, 0.50 g.l^{-1} KH_2PO_4, 0.50 g.l^{-1} $Na_2HPO_4.2H_2O$, 0.17 g.l^{-1} $MgCl_2.6H_2O$ and 0.50 ml.l^{-1} of TES with a final pH of 7.5 (personal communication). Polypropylene glycol was added to the culture medium in all assays in a concentration of 0.15 ml.l^{-1}, as a way to control foam formation. The carbon source was added to obtain a concentration of 10 g.l^{-1} total sugars, unless otherwise stated, and 0.03 g.l^{-1} Na_2SO_4 were added as a S-source. An inoculum of 10% (v.v^{-1}) was used to start the culture and the continuous feed was initiated within a 24 hour time period after inoculation.

Two types of carbon sources were tested to produce the desulfurizing biocatalysts through continuous culture: simple carbon source *versus* sustainable complex carbon source (JAJ). In the majority of the assays, the used carbon source was a mixture of fructose and glucose (8 g.l^{-1} fructose + 2 g.l^{-1} glucose) to mimic the sugar proportion of JAJ, except for the NH_4Cl screening assay where 10 g.l^{-1} of fructose were used. For the preparation of the mixture of simple sugars, both sugars were diluted in Millipore water up to 10% of the total media volume and autoclaved (121°C, 1 atm, 30 min) before being added to the final culture medium. JAJ was filter sterilized using a 0.22 μm pore sterile filter and mixed with the final medium in a laminar flow chamber. The results obtained in the prior shake-flasks minimization assays were used as a basis for the formulations tested in the continuous culture optimization assays. The formulations (#1 to #6) tested in the bioreactors are described in **Table 3**.

All the steady-state cultures of *G. alkanivorans* strain 1B were further characterized through evaluation of their potential for desulfurization of 250 μM of DBT. Several parameters were monitored within the reactor during the steady-state: residual reducing sugars (g.l^{-1}); biomass concentration, expressed as dry cell weight (g.l^{-1}); optical density (OD_{600nm}). The metabolic rates (substrate

consumption, carbon recovery and respiratory quotient) were calculated according to Roseiro *et al.* (1999) and Lopes da Silva *et al.* (2005).

Table 3 – Different formulations of the SFM_R media, with the amounts of the components NH₄Cl, MgCl₂.6H₂O and TES, tested in continuous culture assays towards the design of the optimal minimal culture medium to produce effective desulfurizing biocatalysts.

Set Assays	Steady-state medium formulations (SS/F#)	Dilution rate (h⁻¹)	[a]SFM_R culture medium composition			Carbon source (10 g.l⁻¹)[d]	Sulfur source
			Components minimized				
			NH₄Cl (g.l⁻¹)	MgCl₂.6H₂O (g.l⁻¹)	TES (ml.l⁻¹)		Na₂SO₄ (g.l⁻¹)
N screening[b]	#1	≈0.08	1.22 **(100%)**	0.17	0.50	Fru	0.03
	#2		0.92 **(75%)**				
	#3		0.61 **(50%)**				
	#4		0.31 **(25%)**				
Minimized Medium ([c]SFMM_R)	#5	≈0.07	1.10 **(90%)**	0.04 **(25%)**	0.13 **(25%)**	8Fru + 2Glu	0.03
	#6	≈0.05	1.10 **(90%)**	0.04 **(25%)**	0.13 **(25%)**	JAJ[c]	0

[a]SFM_R – Sulfur-free mineral medium adapted for reactor
[b]Assays in a low-cost prototype bioreactor
[c]SFMM_R – SFM minimum media adapted for reactor
[d]10 g.l⁻¹ total sugars; 8 Fru: fructose 8 g.l⁻¹; 2 Glu: glucose 2 g.l⁻¹; JAJ_c: Jerusalem artichoke juice concentrate
Note: In each continuous culture assay, it was added 0.150 ml.l⁻¹ of PPG were added as anti-foam agent.

2.5.1. BDS assays using resting cells

To evaluate the desulfurization ability, cells were collected during each steady-state of the culture grown in chemostat to a flask and kept on ice, in order to maintain cell properties. The cell samples were then centrifuged in a refrigerated centrifuge (Sigma model 2-16K, Sartorius AG, Germany), using 50 ml centrifuge tubes, at 7673 x *g* for 20 minutes at 4°C. The resulting pellet was washed with a phosphate solution (2.55 g.l⁻¹ KH_2PO_4 and 2.55 g.l⁻¹ $Na_2HPO_4.2H_2O$, pH 7.5) and centrifuged again in the same conditions. Finally, the cells were re-suspended in fresh phosphate solution in order to achieve a final concentration of about 7 g.l⁻¹ (resting cells suspension).

BDS assays were performed in 50 ml shake-flasks with 22 ml of total volume and a concentration of DBT of 250 µM (30°C, 150 rpm). Sampling was performed every 30 min for 4 hours. Additional samples were collected before and after the resting cells desulfurization assays and stored at -20°C, for pigment assessment.

In addition to DBT, a model oil was also tested in desulfurization assays using resting cells from the steady-state selected from the optimal continuous culture assays. The model oil consisted of a mixture of DBT, 4-methyl-DBT, 4,6-dimethyl-DBT and 4,6-diethyl-DBT diluted in o-xylene. This model oil was added to the resting cells in a 1:5 ratio (organic phase to aqueous phase) to obtain a final

concentration of 500 µM of the different DBT, and incubated in closed 100 ml Duran flasks at 30°C and 150 rpm for 24 hours. All BDS assays were performed in triplicates.

2.5.2. Pigments extraction

The biomass samples were centrifuged in a Sigma 2-16K refrigerated centrifuge (7673 x g, 20 minutes, 4°C). Each supernatant was discarded and the pellet was uniformly spread in petri dishes and then dried at 50°C until ~60% water content was obtained. 25 mg of the dried biomass was weighted to 1.5 ml micro centrifuge tubes for extraction, and another 25 mg for dry weight assessment.

Pigments extraction was performed with 1 ml of Dimethyl Sulfoxide (DMSO) (50°C, 1 hour, 150 rpm) in an incubator shaker (Optic ivymen system, Comecta S.A, Spain) and finally the tubes were centrifuged at 15000 x g for 5 minutes (Biofuge 15 centrifuge, Heraeus Sepatech, Germany). The supernatants, containing the pigments, were transferred to tubes protected from the light and another 500 µl of DMSO were added to the respective pellets. After being re-suspended, the samples were incubated for 30 minutes in the conditions described above, and then centrifuged in the same way. This process was repeated until the DMSO was clear.

The resulting DMSO samples were mixed with acetone, NaCl (20% $v.v^{-1}$) and ethyl acetate in a ratio of 4:1:1:6 respectively and softly inverted and kept at -20°C overnight, so that phase separation would occur. The top colored layer was removed and filtered through 0.22 µm syringe filters for the remaining analysis. Samples were protected from direct light at all times and stored at -20°C in order to avoid pigment degradation prior to analysis.

2.6. Analytical methods

2.6.1. Optical density and dry cell weight

The culture growth was monitored by analysis of the optical density at 600 nm (OD_{600nm}) (Thermo Fisher Scientific spectrophotometer, model Genesys 20, USA) and dry cell weight (DCW) values. DCW was determined by centrifuging the samples 10 minutes at 15000 x g at room temperature in a Biofuge 15 (Heraeus Sepatech, Germany) and drying them overnight at 100°C.

2.6.2. Sulfate analysis

Sulfate concentration was determined by high-performance liquid chromatography using a Dionex chromatograph ICS-1000 (Dionex Corporation, Sunnyvale, CA), equipped with an ASRS - Ultra II suppressor, an AG14A (4 x 50 mm) pre-column and an IONPAC AS14A (4 x 250 mm) column. Sodium carbonate 8 mM was used as mobile phase at a flow rate of 1 $ml.min^{-1}$.

Another sulfate assessment technique was used, based in the precipitation with $BaCl_2$. SulfaVer4 powder pillows (HACH Company, Loveland, Colorado, USA) were used accordingly to the HACH DR/2000 spectrophotometer handbook. (HACH Company, Loveland, Colorado, USA).

2.6.3. Desulfurization assessment

DBT and its derivatives desulfurization was evaluated by measuring each respective 2-HBP production, which is the final product of the DBT desulfurization through the 4S-pathway. The samples (0.750 ml) were acidified with 0.025 ml of HCl (4 M) and then a liquid-liquid extraction with ethyl acetate was performed on a vortex (5 minutes), in order to extract 2-HBP and DBT. After phase separation, the organic phase was analyzed by gas-chromatography (GC) (model CP9001, Chrompack, Middelburg, The Netherlands) equipped with a flame ionization detector. A 10% CP-5 CB on 100/120 MESH Chromosorb W-HP column was used with nitrogen as the carrier gas. The chromatograph oven start temperature was 130°C for 3 minutes and the end temperature 230°C maintained for 3 minutes (heating rate of $6°C.min^{-1}$). The injector and detector temperatures were set for 280 and 290°C, respectively. In all GC measurements (except for model oil) 4-methyl-DBT was used as internal standard to minimize variations.

2.6.4. Sugar measurement

Glucose and fructose concentrations were measured by high-performance liquid chromatography (HPLC) instrumentation (LaChrom Merck/Hitachi, Germany) equipped with a differential refractive index detector. A Waters SugarPak 1 column (6.5 x 300 mm, Bio-Rad Laboratories, CA, USA) was used operating at 75°C with Ca-EDTA at 50 $g.l^{-1}$ as mobile phase at a flow rate of 0.5 $ml.min^{-1}$. Data was analyzed using Chromeleon software ver. 6.40 SP6 build 783 (1994-2003, Dionex).

2.6.5. Cell health assessment

Flow cytometric analysis was performed in a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) equipped with an argon laser emitting at 488 nm and data was analyzed using FCS Express 5 Flow Research Edition software (De Novo Software, 2015). *G. alkanivorans* cells were stained with both 5,6-carboxyfluorescein diacetate (CFDA) (green fluorescence, FL1) and Propidium iodide (PI) (red fluorescence, FL3). Stock solutions of 10 mM CFDA in acetone and 1.5 mM PI in distilled water were previously prepared and stored at -20°C and 4°C, respectively. Cells were centrifuged at 8500 x *g* for 10 minutes (Biofuge 15, Heraeus Sepatech, Germany), resuspended in Tris-HCl buffer (50 mM, pH 7.4) and sonicated for 10 seconds. Concentration of the cell suspension was adjusted to approximately 3000 events per second by flow cytometric analysis and the sample total volume set to 0.995 ml. The cells were then incubated with 5 µl of CFDA solution for 30 minutes at 37°C protected from light. After incubation, cells were washed and re-suspended in Tris-HCl buffer and maintained in ice. For the double-staining, cells were adjusted to 1000 events per second and 1 µl of PI solution was added and the flow cytometric analysis performed immediately. Each sample

was collected in duplicate and readings were performed six times for each duplicate. Instrument settings were selected for the forward and side scatter signals in order to discriminate the cells from background noise and debris. Samples containing Tris-HCl buffer without cells were analyzed to confirm the presence of a constant background noise which was not due to unstained cells. For the control assays, healthy (metabolic active) cells were harvested during the exponential phase using fructose as the single carbon source (5 g.l^{-1}) and 200 µM of DBT, and after several days of nutrient starvation (stressed cells). Dead cells were obtained by incubating healthy cells in ethanol at 70% (v.v^{-1}) for 1 min (Silva 2012).

2.6.6. Real Time-PCR assays: Desulfurization gene expression assessment

The evaluation of the desulfurization gene expression was performed using the StepOne Plus Real Time-PCR system (Applied Biosystems). A two-step approach was used: the first step consisted in performing RNA extraction and cDNA formation, followed by amplification and quantification. For RNA extraction, cells of each steady-state were immediately frozen to inhibit cell modification. These samples were then thawed in ice (in order to minimize the temperature shock), normalized to 2 mg of cells and centrifuged at 8000 x g for 5 minutes in a Microfuge 18 centrifuge (Beckman Coulter inc., CA, USA) to remove the growth media. 250 µl of lysozyme (6 mg.ml^{-1} in Tris-EDTA buffer, pH 8) was then added and left to incubate for 1 minute at room temperature. After a new centrifugation at 8000 x g for 2 minutes, the pellet was again suspended in 250 µl of lysozyme and left to incubate at 37°C for 40 minutes. For the next extraction steps, the RNA extraction kit RNeasy MiniKit (Qiagen, Austin, TX, USA) was used, and the supplied protocol was followed. The amount and purity of resulting RNA was accessed using NanoDrop 2000c (Thermo Scientific, USA).

For the production of cDNA from the extracted RNA, the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used and PCR was performed in a C100 Thermal Cycler (Bio-Rad) with the following conditions: 10 minutes at 25°C; 120 minutes at 37°C; 5 minutes at 85°C; 4°C infinitely. The obtained cDNA was then used in the RT-PCR reaction with SYBR-Green PCR master mix (Applied Biosystems). SYBR-Green is a DNA binding fluorophore that has high affinity for double stranded DNA, enabling the quantification of specific genes. In this case, the primers used are referred to the desulfurization genes *dsz*A, *dsz*B, *dsz*C, being the reference gene one copy of 16S DNA (whose expression is highly conserved). The gene sequence and melting points are represented in **Table 4**.

PCR conditions were as follows: 10 minutes at 95°C; 40 cycles of 15 seconds at 95°C and 1 minute at 62°C.

Table 4 – Sequences, melting temperatures and amplification efficiencies of the different dsz operon genes and of the chosen conserved gene used in the RT-PCR analysis.

Gene	Sequence (5'-3')	Melting Temperature (°C)	Amplification Efficiency (E)
*dsz*A	TGGGATTGATGCAGGCTACAT	56.5	2.05
	GCCCCGCAGCCTTCAC	60.3	
*dsz*B	CCTGCTGGATCGCACACA	58.1	1.89
	CGGGCTCCTGCAGCAA	58.9	
*dsz*C	TGTTCGGCTCGCAGGAA	57.1	1.99
	CGTTCTGCGCGATTTGC	55.4	
16S rRNA	CGACCTGAGAGGGTGATC	57.1	1.89
	ATAACCCGCTGGCAATACAG	55.2	

2.6.7. Pigments quantification and analysis

Pigments quantification was performed using HPLC (Agilent 1200 Series system, Agilent Technologies, Tokyo) equipped with a µ-Bondapack C18 (250/4.6 mm) column and a detector UV/VIS also from Agilent (λ=477 nm). The mobile phase was composed by methanol (with 0.2% v.v^{-1} water) and acetonitrile in a 75:25 ratio in a 0.500 ml.min^{-1} flow during 90 min. Samples were then analyzed and compared with standards for astaxanthin (Sigma, 98% purity), canthaxanthin (Roche, 99% purity) and lutein (FloraGLO, Kemin, 95% purity) using LC3D ChemStation software (Rev.A.10.02 [1757], 1990-2003, Agilent Technologies, USA).

3. Results and discussion

In an industrial bioprocess it is very important to control all the stages in order to maximize productivity and minimize the production costs. The reduction of production costs can be achieved through the utilization of cheaper carbon sources and by reducing nutrient waste to a minimum (Min *et al.* 2011; Jiang *et al.* 2012; Lu *et al.* 2013; Abo-State *et al.* 2014). This study consisted on the optimization of the SFM culture medium for the maximum production of efficient desulfurizing biocatalysts (*G. alkanivorans* strain 1B active cells) taking into account the lowest operation costs towards a future BDS process scale-up. In this context, key components of the SFM medium used to grow strain 1B (NH$_4$Cl, MgCl$_2$ and TES) were reduced to a minimum while aiming to have no limitation both on the consumption of the carbon source and on the biodesulfurization ability.

3.1. Culture medium minimization in shaken flask assays

The first series of assays towards the *G. alkanivorans* strain 1B culture medium minimization were carried out in shake-flask. In fact, different formulations of the SFM medium, with different amounts of the components: NH$_4$Cl, MgCl$_2$.6H$_2$O and TES (F#1 to F#15), as detailed in **Table 2**, were tested towards the design of the optimal minimum culture medium to produce effective desulfurizing biocatalysts.

3.1.1. Nitrogen source (NH₄Cl)

Nitrogen (N) is a major intervenient in cellular growth, representing 14% of the dry cell weight. It is found in the composition of amino acids, nucleic acids and enzymes/coenzymes, therefore, it is one of the major macronutrients of most culture media (Singh 1971).

Hence, in the first set of BDS assays in shake-flask, three formulations with different amounts of N-source (NH₄Cl) were tested, namely 75% (F#2), 50% (F#3) and 25% (F#4) of the amount in the original medium (1.22 g.l^{-1} = 100% N), simultaneously with the original medium as a control (F#1 – default SFM medium), for comparison. In these screening assays for the N-source minimization (see **Table 2**), the carbon source used was a mixture of fructose and glucose (8 g.l^{-1} Fru + 2 g.l^{-1} Glu) and the sulfur source was 250 µM DBT.

Figure 9 (A,B) shows the time course profiles of cellular growth (OD$_{600nm}$) and sugar consumption for the formulations F#1 to F#4, with the different NH₄Cl concentrations tested (100% N (original SFM) to 25% N).
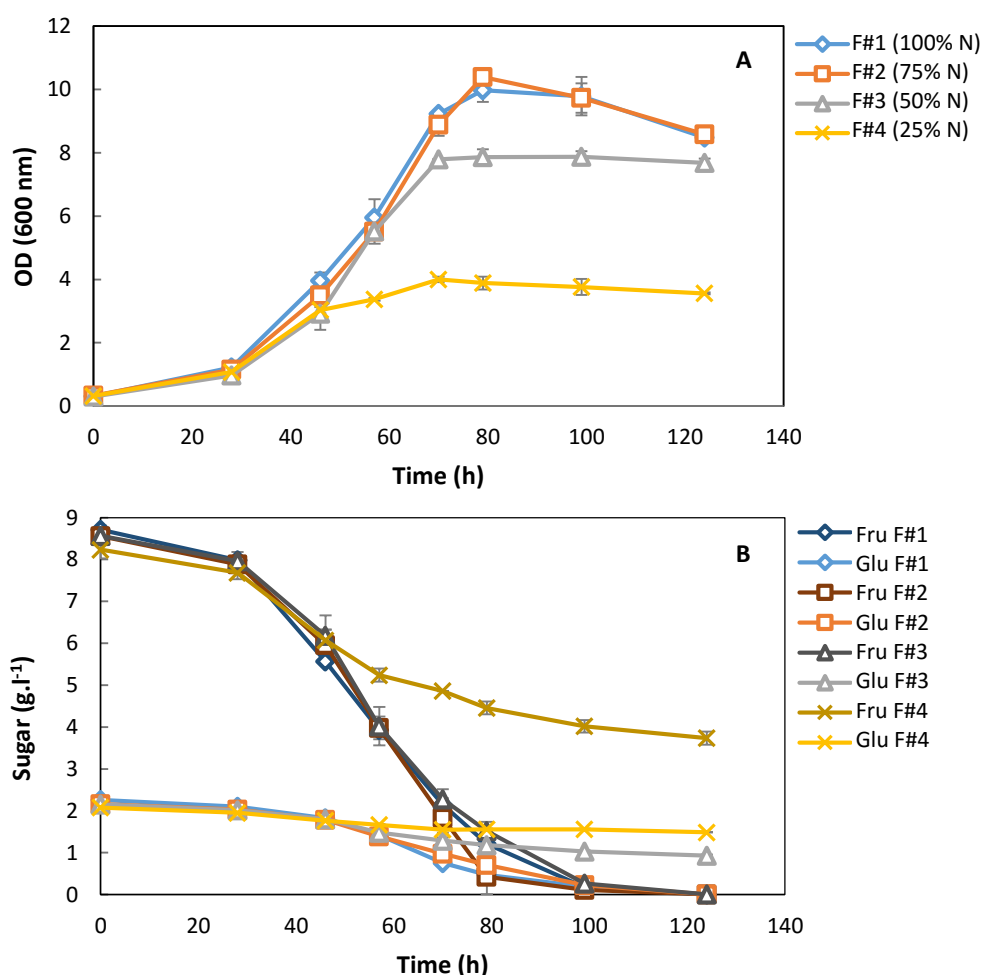
Figure 9 *– Time course profiles for cellular growth (A) and sugar consumption (B) of* G. alkanivorans *strain 1B in the shake-flask cultures with different medium formulations (F#1 to F#4) containing decreasing concentrations of N-source (NH₄Cl). F#1 corresponds to the control assay with the original SFM medium. All formulations contain 10 g.l^{-1} total sugars as carbon source. Fru: fructose; Glu: glucose.*
Standard deviation (n = 3) is indicated by the error bars.

These results show that the bacterial culture attained an OD_{600nm} of 9.98 with complete consumption of the carbon source (Fru+Glu) in the original medium (F#1 – 100% N); an OD_{600nm} of 10.39 with also complete consumption of both sugars in F#2 (75% N); an OD_{600} of 7.87 with complete consumption of the fructose but with a remaining of 0.9 g.l^{-1} of glucose in F#3 (50% N); and an OD_{600nm} of only 4 with more than half of the total sugars (3.7 g.l^{-1} Fru and 1.5 g.l^{-1} Glu) remaining intact in F#4, which had the lowest concentration of NH_4Cl tested (25% N).

The data indicates that the amount of the N-source greatly influenced the bacterial behavior. F#4 (25% N) was clearly insufficient to ensure normal cell growth, resulting in an abrupt stop of the growth when all other nutrients were still in excess. Relatively to F#4, F#3 (50% N) permitted a growth enhancement, almost doubling the maximum OD_{600nm} attained, but without achieving full consumption of sugars. In F#3, fructose was fully consumed but most of the glucose was left untouched. This preferential consumption of fructose was expected since the strain 1B is fructophilic (Alves & Paixão 2014b). Only in F#2 (75% N), the strain 1B was presented a growth curve similar to the one obtained with 100% N (F#1, original medium), also with a complete consumption of the carbon source (Fru+Glu).

The desulfurization ability of the strain 1B depends mostly on cell physiology, which is influenced by the amount of nutrients and carbon in the culture medium. **Figure 10** shows the DBT desulfurization by strain 1B grown in the formulations F#1 to F#4, with decreasing amounts of N-source (100% – 25%).
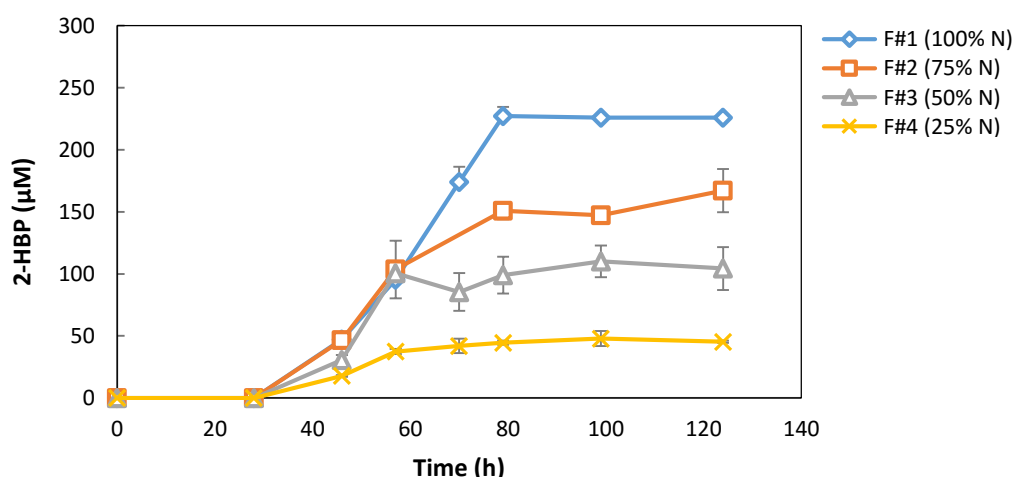

Figure 10 – Desulfurization curves (2-HBP production) by G. alkanivorans strain 1B during its growth in different medium formulations (F#1 to F#4) containing decreasing concentrations of N-source (NH_4Cl). F#1 corresponds to the control assay (SFM default medium). All formulations contained 250 µM DBT as sulfur source. Standard deviation (n = 3) is represented as error bars.

These results point out for the limitation of desulfurization ability of strain 1B by the lack of N-source in the culture medium. In F#1, the bacterial culture presented a maximum 2-HBP production of 227.2 µM and a maximum specific 2-HBP production rate ($q_{2\text{-HBP}}$) of 2.5 µmol.g^{-1}(DCW).h^{-1}, being capable of achieving total consumption of the 250 µM of DBT by the end of the growth. In F#2, the

$q_{2\text{-HBP}}$ obtained was 2.4 µmol.g^{-1}(DCW).h^{-1} with a maximum 2-HBP of 167.1 µM and a remaining 27 µM of DBT. In F#3 and F#4, the $q_{2\text{-HBP}}$ obtained was 2.25 µmol.g^{-1}(DCW).h^{-1} and 1.41 µmol.g^{-1}(DCW).h^{-1}, and the maximum 2-HBP produced was 110.1 µM and 47.8 µM, respectively. Relatively to DBT, 69 µM were not consumed in F#3 and 202 µM were not consumed in F#5. The desulfurization results for both F#3 and F#4 formulations can be supported by their respective results for cellular growth and sugar consumption, showed in **Figure 9 (A, B)**.

In fact, a NH$_4$Cl concentration $\leq$75% of the original amount (F#2 to F#4) seems to not be sufficient for strain 1B to achieve maximum growth with total C-source consumption and fully desulfurization of the 250 µM DBT provided. The overall growth, sugar consumption profiles and also the $q_{2\text{-HBP}}$ were very similar for F#1 and F#2, however a maximum 2-HBP production of 167.1 µM was observed in F#2. In contrast, in F#1 the maximum 2-HBP production was 227.2 µM (~60 µM higher), despite of the consumption of almost all DBT (223 µM) in the F#2. Hence, these results point to the need of using a concentration of NH$_4$Cl >75% of the original amount as the minimal N-source towards a minimized SFM medium.

Since nitrogen is a major component of enzymes and functions as a co-factor for innumerous essential proteins (Singh 1971; Schimel & Weintraub 2003), it is expected that insufficient amounts will lead to deficiencies in cellular processes. This fact can support the sequential decrease in the desulfurization rate and, consequently, the maximum 2-HBP production observed in the formulations tested, namely from F#1 (100% N) to F#4 (25% N). Only in F#1 (default medium), the N amount was sufficient to support the complete cell growth, using the totality of C-source available, and the biodesulfurization of all the DBT. In the other formulations (F#2 to F#4), with decreasing NH$_4$Cl concentrations, increasing limitations at different levels (e.g. growth rate, sugar consumption, 2-HBP production or $q_{2\text{-HBP}}$) were observed.

Therefore, based on results from **Figures 9B and 10**, 85% N was extrapolated as an appropriate amount to be further tested as minimal N-source within a SFM minimized medium (F#14 and F#15 in **Table 2**).

3.1.2. Magnesium source (MgCl$_2$.6H$_2$O)

Magnesium (Mg) is an element essential for cell division and protoplasm formation and is especially important for gram-positive bacteria, since it is incorporated in the structure of the peptidoglycan which is 90% of the cellular wall (Webb 1939). The SFM medium usually used for the growth of *G. alkanivorans* strain 1B has 0.17 g.l^{-1} of MgCl$_2$.6H$_2$O (100% Mg). So, as for the N-source amount minimization assays, to select the minimal amount of Mg-source that supports bacterial growth and desulfurization ability, several formulations with decreasing concentrations of Mg-source were tested (F#5 to F#7, as represented in **Table 2**), and indicated as percentage of the original amount. The

results obtained were compared with those of 100% Mg (F#1, control assay with default SFM medium).

Figure 11 (A,B) shows the time course profiles of cellular growth (OD_{600nm}) and sugar consumption for the medium formulations with the different Mg-source concentrations tested: F#1 (100% Mg) (control with original medium), F#5 (75% Mg), F#6 (50% Mg) and F#7 (25% Mg). These results show that strain 1B growth and sugar consumption profiles for F#5 and F#6, were very similar to those observed for F#1. A total consumption of the 10 g.l^{-1} total sugars, within 120 h, was observed for these three formulations achieving an OD_{600nm} around 10. However, when F#7 was used as culture medium, the strain 1B only attained a maximum OD_{600nm} of 8.66 with an overall growth profile slightly below the others, despite having a similar growth rate ($\mu_{Max} \sim 0.06$ h^{-1} to all). Moreover, strain 1B in F#7 medium consumed all the C-source (Fru+Glu) with the same sugar consumption profile as the other formulations. This fact contrasts with what was observed when N-source was limiting, where accumulation of sugars was detected.
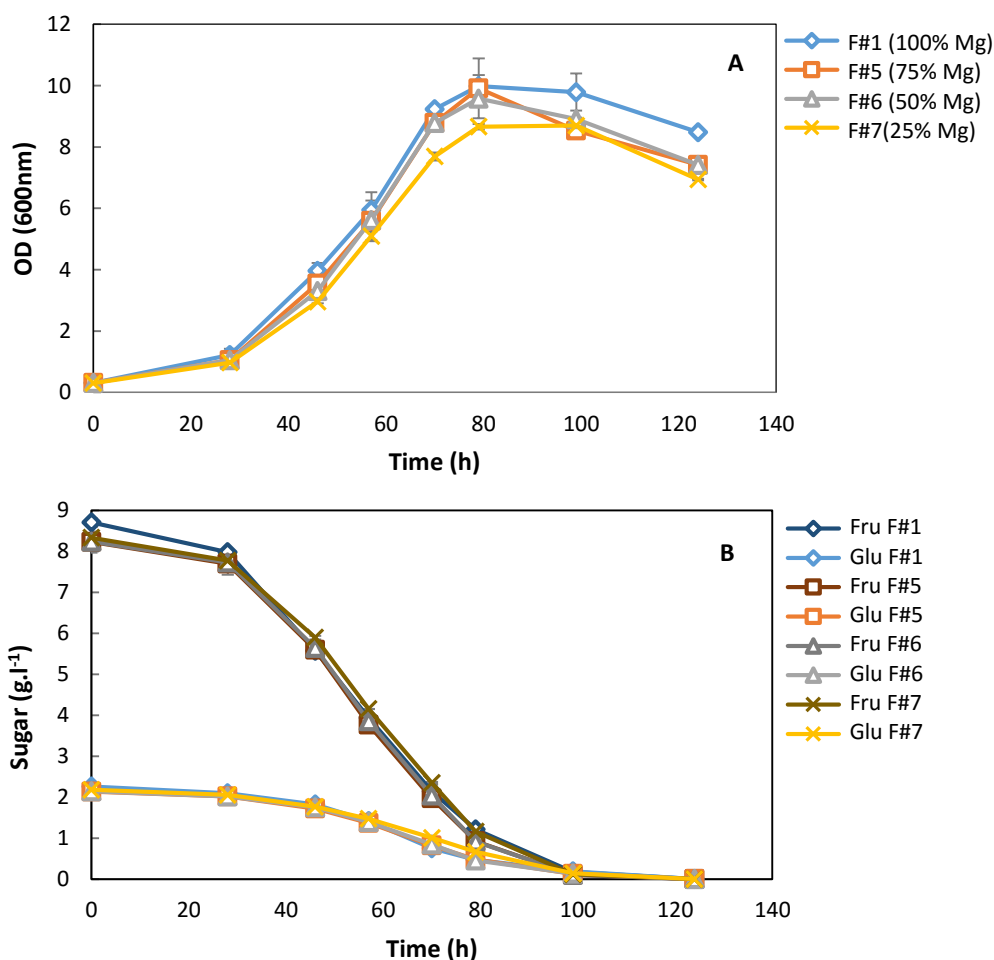
Figure 11 – Time course profiles for cellular growth (A) and sugar consumption (B) of G. alkanivorans strain 1B in the shake- flask cultures with different medium formulations (F#1, F#5 to F#7) containing decreasing concentrations of Mg-source (MgCl$_2$.6H$_2$O). F#1 corresponds to the control assay (100% Mg in SFM default medium). All formulations contained 10 g.l^{-1} total sugars as carbon source. Fru: fructose; Glu: glucose.
Standard deviation (n=3) is represented as error bars.

Figure 12 presents the desulfurization curves (2-HBP production) by the strain 1B grown in the different Mg-source medium formulations. As already described in the N-source minimization assays, for control formulation (F#1) a $q_{2\text{-HBP}}$ of 2.45 µmol.g^{-1}(DCW).h^{-1} and a maximum 2-HBP of 227.2 µM were achieved. For the other formulations tested, the respective $q_{2\text{-HBP}}$ and maximum 2-HBP obtained were: 2.67 µmol.g^{-1}(DCW).h^{-1} and 252.2 µM (F#5), 3.01 µmol.g^{-1}(DCW).h^{-1} and 266.4 µM (F#6), and 3.37 µmol.g^{-1}(DCW).h^{-1} and 272.1 µM (F#7). In all these formulations, strain 1B was capable of fully desulfurize the DBT provided.
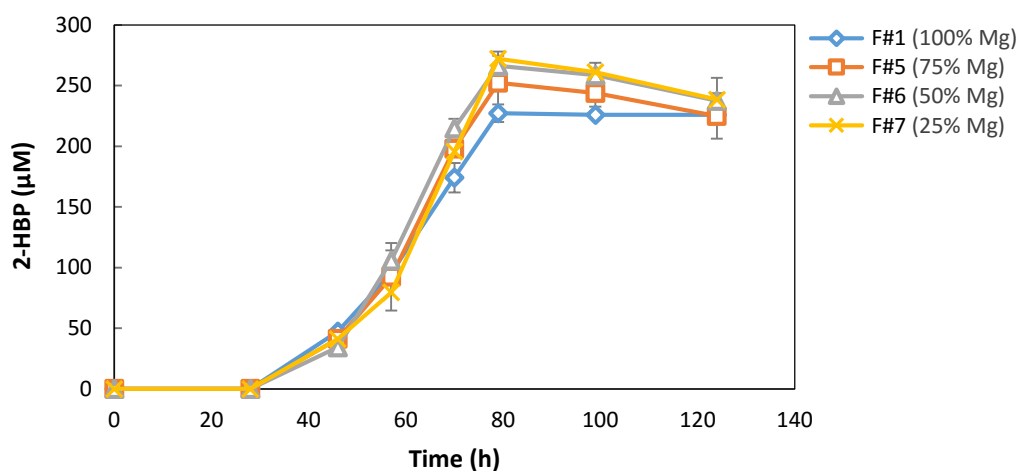

Figure 12 – Desulfurization curves (2-HBP production) by <u>G. alkanivorans</u> strain 1B during its growth in different medium formulations (F#1, F#5 to F#7) containing decreasing concentrations of Mg-source (MgCl$_2$.6H$_2$O). F#1 corresponds to the control assay (SFM default medium). All formulations contained ~250 µM DBT as sulfur source.
Standard deviation (n = 3) is represented as error bars.

These results show an evident enhancement of the maximum specific desulfurization rate ($q_{2\text{-HBP}}$) with the decrease of the Mg-source:C-source ratio, from 2.45 µmol.g^{-1}(DCW).h^{-1} (ratio 100% Mg:10 g.l^{-1} C in F#1) to 3.37 µmol.g^{-1}(DCW).h^{-1} (ratio 25% Mg:10 g.l^{-1} C in F#7). This fact is in agreement with results previously reported in studies of DBT desulfurization by strain 1B (Silva 2012; Alves & Paixão 2014b). These authors reported a $q_{2\text{-HBP}}$ of 2.12 µmol.g^{-1}(DCW).h^{-1} by strain 1B when SFM medium with a ratio 100% Mg:10 g.l^{-1} Fru was used and a $q_{2\text{-HBP}}$ of 6.57 µmol.g^{-1}(DCW).h^{-1} by strain 1B when SFM medium with a ratio 100% Mg:25 g.l^{-1} Fru was used. These results also pointed out for the stimulatory effect on desulfurization by the decrease of Mg-source:C-source ratio.

Hence, F#7 (25% Mg) allowed a similar maximum growth rate, despite the lower value for maximum OD$_{600nm}$, and the enhancement of desulfurization. Therefore, it was stipulated as an appropriate amount to be further tested as minimal Mg-source within a SFM minimized medium.

3.1.3. Trace Element Solution (TES)

Trace elements are minerals, such as iron, cobalt and zinc, which are important co-factors in the constitution of enzymes (Schade 1949; Waldron & Robinson 2009; Majzlik *et al.* 2011; Kirsch & Eitinger 2014). In fact, 25% – 33% of all proteins require metals in its constitution. These compounds are necessary in relatively small amounts, often too small to be correctly measured. Usually, they are

added to the culture media in the form of a solution that includes all the trace elements needed (Alves, Matos, *et al.* 2008).

For the SFM medium used to grow *G. alkanivorans* strain 1B, a sulfur-free TES stock solution (Alves & Paixão 2014b) is usually added in a concentration of 0.5 ml.l^{-1}. Therefore, as in prior assays performed for N and Mg sources minimization, different formulations with decreasing concentrations of TES (F#8 to F#13, represented in **Table 2**) were tested and compared with control (100% TES, F#1) in order to evaluate the minimum concentration of the TES needed by the bacterium to grow and desulfurize DBT.

Figure 13 (A,B) presents the cellular growth (OD$_{600nm}$) and sugar consumption profiles for strain 1B grown on the different culture medium formulations containing decreasing concentrations of TES (100% to 0%). In **Figure 13B**, only the sugar consumption profiles for the formulations that significantly differ from control (F#1) are represented.
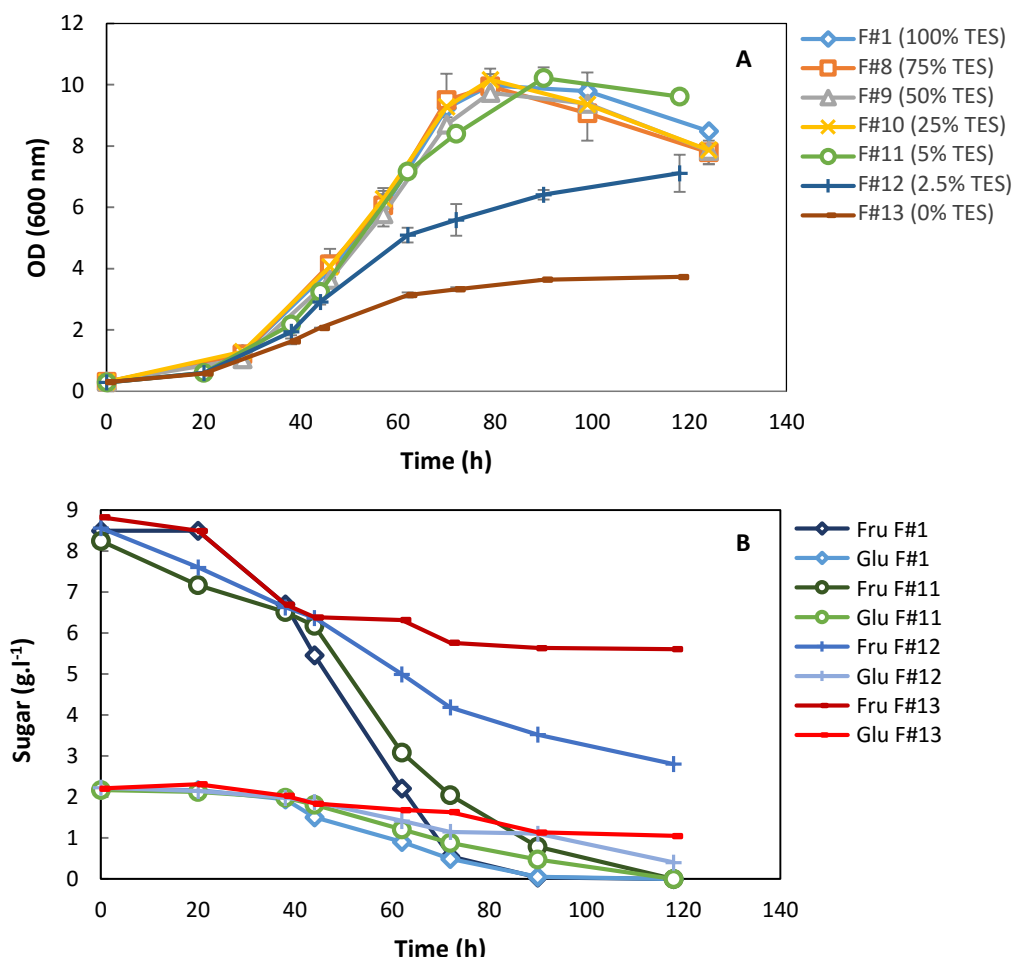
***Figure 13** – Time course profiles for cellular growth (A) and sugar consumption (B) of G. alkanivorans strain 1B in the shake-flask cultures with different medium formulations (F#1, F#8 to F#13) containing decreasing concentrations of TES. In graphic B are only represented F#1 and F#11 to F#13. F#1 corresponds to the control assay with the original SFM medium. All formulations contain 10 g.l^{-1} total sugars as carbon source. Fru: fructose; Glu: glucose. Standard deviation (n = 3) is indicated by the error bars.*

These results show that the media formulations F#1 (100% TES), F#8 (75% TES), F#9 (50% TES) and F#10 (25% TES) induced a similar behavior on strain 1B and consequently the growth curves and the sugar consumption profiles were identical. So, as for the control (F#1) and for F#8 to F#10 formulations, a maximum OD_{600nm} around 10 was attained with a total sugar consumption (10 g.l^{-1}) within 124 h. This fact indicates that TES $\geq$ 25% of the original amount is still in excess.

So, in order to find the limiting concentration where cellular development would be affected, additional formulations with lower TES concentrations, namely 5% (F#11), 2.5% (F#12) and 0% (F#13) were also tested. The results obtained showed that in the absence of the TES (F#13) the maximum OD_{600nm} achieved by strain 1B was only 3.73 with an accumulation of 6.6 g.l^{-1} of sugars. In F#12 (2.5% TES), the bacterium was able to attain an OD_{600nm} of 7.11 but still accumulated 3.2 g.l^{-1} of total sugars. However in F#11 (5% TES), a maximum OD_{600nm} of 10.22 with a total sugar consumption was achieved, similarly to that observed for medium formulations with $\geq$ 25% TES (F#1, F#8 to F#10). These results indicate that a TES concentration $\geq$ 5% is required for maximum growth of strain 1B and full C-source consumption. However, when 5% TES was used the sugar consumption rate was somewhat slower than for $\geq$ 25% TES, prolonging the fermentation from 90 – 100 h to a total of 118h.

Figure 14 presents the desulfurization results as the time course profiles of 2-HBP production by strain 1B cultivated in the different formulations with decreasing TES concentrations.
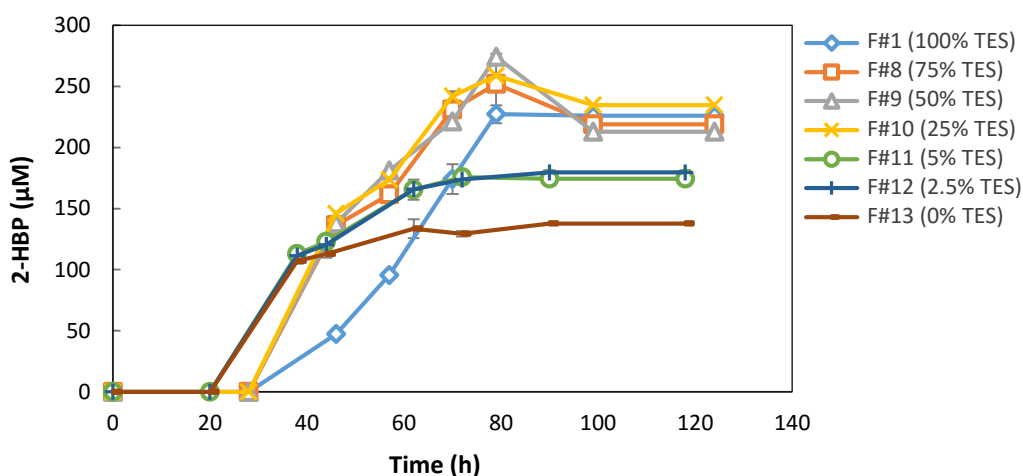
Figure 14 – Desulfurization curves (2-HBP production) by G. alkanivorans strain 1B during its growth in different medium formulations (F#1, F#8 to F#13) containing decreasing concentrations of TES. F#1 corresponds to the control assay (SFM default medium). All formulations contained ~250 µM DBT as sulfur source.
Standard deviation (n = 3) is represented as error bars.

These results show a desulfurization enhancement with the decrease of TES concentration to 25% in the culture medium. However, for concentrations <25% TES, namely in F#11 (5%), F#12 (2.5%) and F#13 (0%), the reduction of trace elements was limiting and the desulfurization stopped within about 60 h, attaining lower levels of 2-HBP production. At 0% TES the strain 1B desulfurization was probably mediated by trace elements available from water or from contaminants within the other nutrients used in the culture medium.

Table 5 summarizes the main metabolic parameters (maximum $q_{2\text{-HBP}}$ and maximum 2-HBP produced) for the DBT desulfurization by *G. alkanivorans* strain 1B grown in the formulations with decreasing TES concentrations. In agreement with the results presented in **Figure 14**, the $q_{2\text{-HBP}}$ values for F#8 (2.80 $\mu mol.g^{-1}(DCW).h^{-1}$), F#9 (3.19 $\mu mol.g^{-1}(DCW).h^{-1}$) and F#10 (3.02 $\mu mol.g^{-1}(DCW).h^{-1}$) were higher than the value of the control (F#1, 2.45 $\mu mol.g^{-1}(DCW).h^{-1}$).

Table 5 – Main metabolic parameters for the DBT desulfurization by G. alkanivorans strain 1B in the formulations with decreasing TES concentrations (100% - default medium to 0%).

Media formulations	F#1 (100% TES)	F#8 (75% TES)	F#9 (50% TES)	F#10 (25% TES)	F#11 (5% TES)	F#12 (2.5% TES)	F#13 (0% TES)
$q_{2\text{-HBP}}$ ($\mu mol.g^{-1}(DCW).h^{-1}$)	2.45	2.80	3.19	3.02	1.32	1.26	0.68
2-HBP produced (μM)	227.2	251.9	274.1	258.8	179.5	173.7	133.5

Overall, these results seem to indicate that there is some inhibitory effect caused by the TES, since a sequential decrease of TES concentration up to 25% (F#10) produces cells with higher desulfurization ability than the control. In fact, F#10 with 25% TES induces an enhancement of about 23% of the specific desulfurization rate in comparison with the control (100% TES). These results are also in accordance with what was reported by Alves *et al.* (2008), which described that some of the compounds present in the original TES are somewhat toxic to *G. alkanivorans* strain 1B. They observed that without molybdenum, the strain 1B was able to grow from an OD_{600nm} of 9.25 to 10.25, indicating that this component may possess an inhibitory effect.

Nevertheless, most of the trace elements are essential for strain 1B growth. So the reduction of these micronutrients may result in a poor microbial growth and consequently cause the incomplete desulfurization of the DBT present in culture medium. This can be due to the malformation of essential enzymes, such as DszA, that need the nutrients supplied in the TES to function properly (Ohshiro *et al.* 1999).

Since 25% TES (F#10) was the lowest concentration tested that permitted maximum cell growth and in addition had stimulated desulfurization, this was the chosen amount as the most appropriated to be further tested as minimal TES within a SFM minimized medium.

3.1.4. Minimized medium: commercial *versus* alternative C-source

Using the N-source, Mg-source and TES minimal concentrations selected from the prior set of assays, namely 85% N, 25% Mg and 25% TES, a SFM minimum (SFMM) medium was designed (F#14, **Table 2**) and tested in comparison with the control (F#1 – original SFM medium) to evaluate the overall effect of the nutrients reduction. However, since the main goal of this study was to reduce the costs associated to DBT desulfurization process by *G. alkanivorans* strain 1B, *i.e.* to reduce its culture medium costs, the use of a mixture of commercial sugars (Fru+Glu) as C-source is not the cheapest

option. Silva (2012) and Silva *et al.* (2015), studying alternative carbon sources, highlighted the great potential of JAJ as a sustainable alternative carbon source, which enhances the DBT desulfurization ability of strain 1B. In this context, another SFMM medium formulation was tested using the JAJ as C-source (F#15, **Table 2**). In addition, the respective control was also prepared for comparison (F#1'- original SFM medium with JAJ_p as C-source, **Table 2**). Each control assay, using the original SFM medium, ran simultaneously with the respective minimized medium (SFMM medium) and served to evaluate the overall effect of the collective reduction of several nutrients on metabolic parameters in study (cell growth and desulfurization ability).

Figure 15 (A,B) presents the cellular growth (OD_{600nm}) and sugar consumption profiles for strain 1B grown in both SFMM medium (designed F#14 and F#15) and in their respective controls (F#1 and F#1'), while **Figure 16** presents the biodesulfurization curves obtained with those formulations. In all medium formulations were used 10 g.l^{-1} of total sugars (Fru+Glu or JAJ_p) as carbon source and 250 µM DBT as sulfur source.
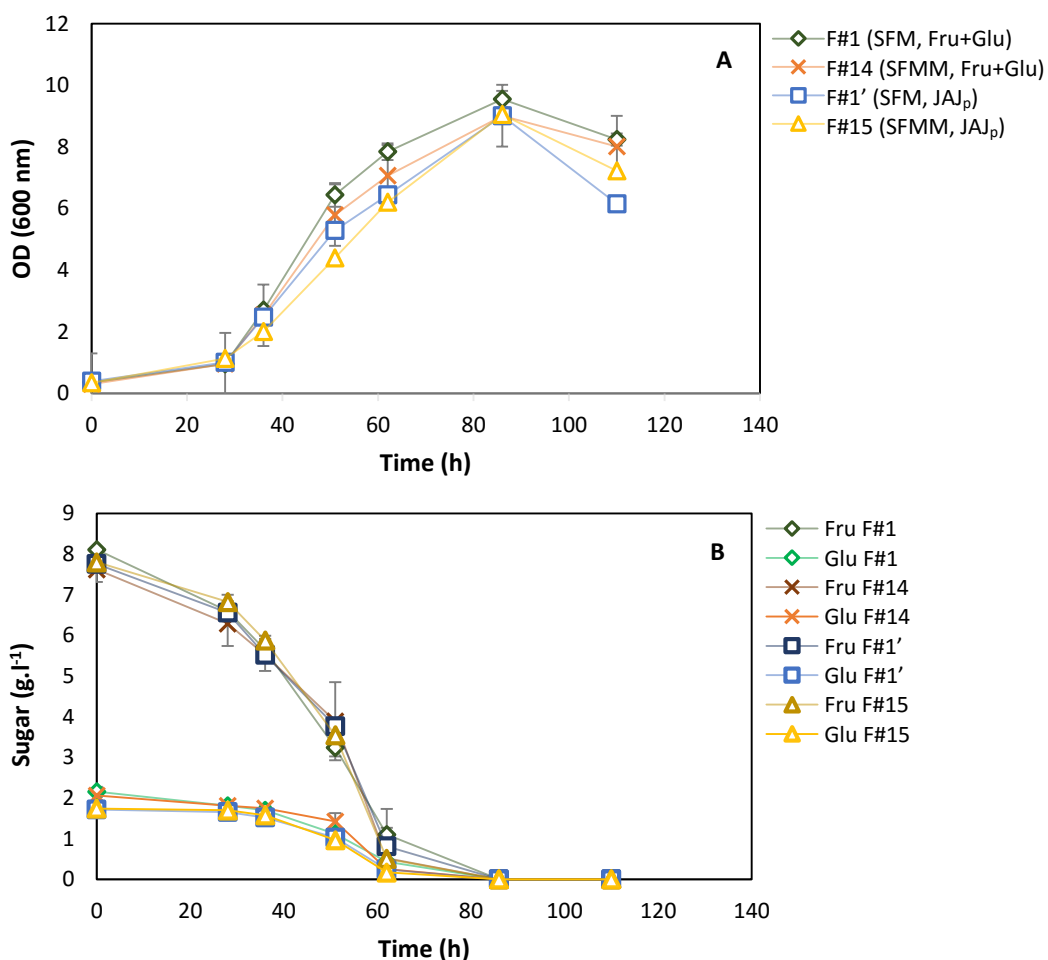
Figure 15 – Time course profiles for cellular growth (A) and sugar consumption (B) of G. alkanivorans strain 1B in the shake-flask cultures with the two SFMM medium formulations containing 10 g.l^{-1} total sugars as C-source, Fru+Glu (F#14) or JAJ_p (F#15), and their respective controls with the original SFM medium and the same C-sources (F#1, F#1'). Fru: fructose; Glu: glucose. Standard deviation (n = 3) is indicated by the error bars.

Comparing the growth profiles (**Figure 15A**) it can be observed that, despite of the different growth rates, strain 1B attained a maximum OD_{600nm} of ~9 in all cultures within 86 h, with the highest OD_{600nm} value observed in F#1 (9.55). Higher growth rates can be observed for both media with Fru+Glu as C-source (F#1-SFM and F#14-SFMM), however in all formulations the sugar consumption presented similar profiles with total consumption within 86 h (**Figure 15B**).
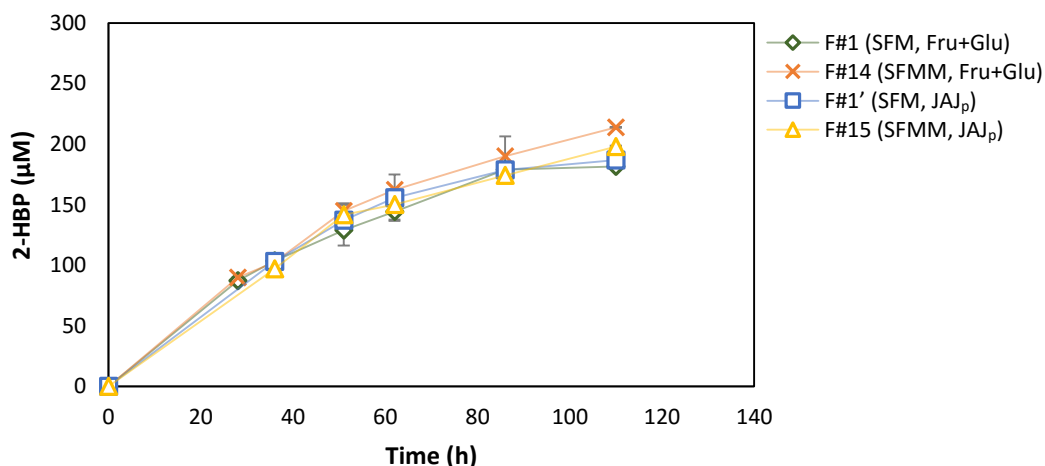

Figure 16 – Desulfurization curves (2-HBP production) by G. alkanivorans strain 1B during its growth in two SFMM medium formulations containing 10 g.l^{-1} total sugars as C-source, Fru+Glu (F#14) or JAJ$_p$ (F#15), and their respective controls with the original SFM medium and the same C-sources (F#1, F#1'). All formulations contained ~250 µM DBT as sulfur source. Fru: fructose; Glu: glucose.
Standard deviation (n = 3) is represented as error bars.

Based on the results from **Figures 15 and 16**, the main metabolic parameters associated to the growth and desulfurization by strain 1B in these four tested formulations (F#1 *vs* F#14; F#1' *vs* F#15) are summarized in **Table 6**.

Table 6 – Main metabolic parameters for the DBT desulfurization by G. alkanivorans strain 1B in the minimized medium formulations with different C-source versus their respective controls: F#1 – SFM, F+G vs F#14 – SFMM, F+G; and F#1' – SFM, JAJ$_p$ vs F#15 – SFMM, JAJ$_p$.

Medium formulations	Fru+Glu		JAJ$_p$	
	F#1* - SFM	F#14 - SFMM	F#1' - SFM	F#15 - SFM
Max. OD_{600nm}	9.55	9.01	9.01	9.05
μ_{Max} (h^{-1})	0.080	0.075	0.069	0.058
Max. q_{2-HBP} (µmol.g^{-1}(DCW).h^{-1})	2.17	2.69	2.10	2.27
Max. 2-HBP produced (µM)	186.7	213.7	181.5	197.9
Max. DBT consumed (µM)	250	250	194	250

Max. – Maximum

JAJ$_p$ – JAJ precipitated with $BaCl_2$ for sulfate removal

*This was a new F#1 control that was repeated in simultaneously with the assays for F#14; F#1' and F#15.

These results demonstrate that independently of the C-source used, the minimized medium (SFMM medium) enhanced the desulfurization by strain 1B when compared to the respective control (SFM medium). This is in accordance with the Mg-source and TES preliminary minimization assays results, since each minimal amount increased desulfurization. Strain 1B grew normally in the minimized medium, and no synergy effects were observed. Comparing the results by C-source (i.e. F#1 *vs* F#1'; F#14 *vs* F#15), it is possible to observe that for both media (SFM and SFMM) either the growth rates (μ_{Max}) or the desulfurization (2-HBP production or $q_{2\text{-HBP}}$) were lower when JAJ was used as carbon source. This behavior was not expected (Alves & Paixão 2014b; Silva *et al.* 2015), however the decrease in both growth and desulfurization ability, when the JAJ$_p$ was used as C-source, may be justified by the potential toxic effect of the residual barium chloride left in the juice after sulfate precipitation procedure. This is in accordance with what was already referred by Silva *et al.* (2012).

3.2. Minimum culture medium: optimization in chemostat

In order to design a future scale-up of the BDS process by *G.alkanivorans* strain 1B, it is necessary to know very well the microbial behavior and physiology in continuous culture. This knowledge allows the choice of the best culture conditions towards a cost-effective industrial process with the highest production rate of biocatalysts.

A chemostat is a system into which fresh culture medium (feed) is continuously added, while spent culture media, containing leftover nutrients, metabolic end products and microorganisms, is continuously removed (effluent) at the same rate to keep the microbial culture volume constant. One of the most important features of chemostats is that microorganisms (bacteria) can be grown in a physiological steady-state under constant and optimal conditions. In steady-state, the growth occurs at a constant specific growth rate and all the culture parameters remain constant (culture volume, temperature, pH, cell density, aeration, agitation, nutrients, products, etc). The microbial specific growth rate can be optimal if the best dilution rate and culture conditions are established *(*Roseiro *et al.* 1996; Roseiro *et al.* 1999; Bandyopadhyay *et al.* 2013).

In this context, based on the minimal amounts of N, Mg and TES tested in the SFMM medium for the shake-flask assays, respectively 85% N, 25% Mg and 25% TES, the next step was to apply this combined reduction in the SFM$_R$ medium and optimize the culture conditions towards the maximum production of efficient desulfurizing biocatalysts, *i.e.* resting cells of strain 1B. Resting cells differ from growing cells because there are no changes in cell metabolism and no cell multiplication, since no nutrients and carbon source are provided. This procedure allows the testing of the desulfurization ability with a constant cell concentration.

3.2.1. Optimization of the N-source minimization in a low-cost chemostat

The first set of assays in chemostat were carried out towards the minimization of the N-source (NH_4Cl) in the SFM_R medium, since the minimal amount of 85% N used in SFMM medium for shake-flask was selected based only in an extrapolation from the sugar and desulfurization results. For these assays, a tailor-made bioreactor system coupling a GLS 80® stirred reactor with 260 ml of working volume was developed. **Figure 17** shows the setup of this low-cost chemostat during an assay of continuous culture with strain 1B for N-source minimization. In this system, the most expensive parts are the pH controller, the pH electrode (250 mm), the peristaltic pumps and the GLS 80® stirred reactor. The other components can be easily found in a laboratory.
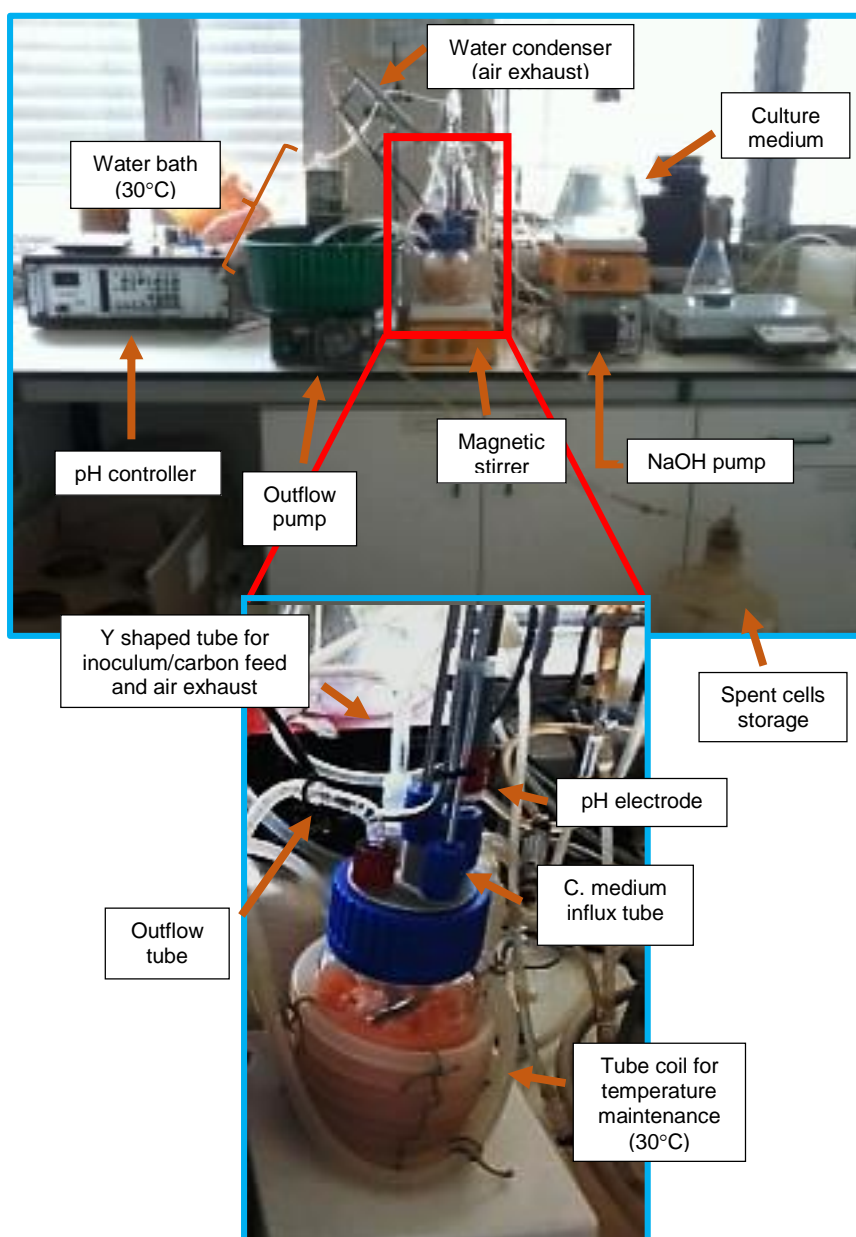

Figure 17 – Setup of the low-cost chemostat during an assay of continuous culture with strain 1B.

Therefore, different medium formulations with decreasing concentrations of N-source, F#1 (100%), F#2 (75%), F#3 (50%) and F#4 (25%) (see **Table 3**), were tested in order to evaluate the minimum concentration of NH_4Cl needed by the bacterium to grow and further desulfurize. In these continuous cultures, the only C-source used was 10 $g.l^{-1}$ fructose, since it allows the use of higher dilution rates (0.07– 0.08 h^{-1}) and consequently decreases the time necessary to enter steady-state. **Table 7** presents the metabolic characteristics for each bacterial culture steady-state (SS) obtained for the formulations F#1 to F#4, designated as SS#1, SS#2, SS#3 and SS#4, respectively. All conditions were maintained constant; therefore the culture behavior translates the physiological response of the microbial cells to the influence of the N-source amount in the culture medium.

Table 7 – *Metabolic parameters for the steady-state cultures of* G. alkanivorans *strain 1B, grown in a low-cost chemostat in SFM_R medium formulations with decreasing N-source concentrations.*

Steady-state culture (SS#) (N-source amount[a])	SS#1 (100%)	SS#2 (75%)	SS#3 (50%)	SS#4 (25%)
Dilution rate (h^{-1})	0.076	0.072	0.076	0.082
Biomass production rate ($g.l^{-1}.h^{-1}$)	0.37	0.32	0.22	0.09
Fructose consumption rate ($g.l^{-1}.h^{-1}$)	0.76	0.64	0.48	0.15
q_{Fru} (mmol.g^{-1} (DCW).h^{-1})	0.87	0.80	0.92	0.77
q_{SO4} (mmol.g^{-1} (DCW).h^{-1})	3.04	3.31	3.76	3.22
q_{CO2} (mmol.g^{-1} (DCW).h^{-1})	1.24	1.23	1.30	1.62
CR (%)	84.93	87.76	81.43	110.57
CCE (%)	61.02	62.31	57.80	74.70
$Y_{x/s}$ (g (DCW).mol^{-1})	87.80	89.66	83.16	107.48
Accumulated carbon source ($g.l^{-1}$)	0.00	1.07	3.69	8.15
OD_{600nm}	12.63	13.21	7.79	3.46
Biomass ($g.l^{-1}$)	4.83	4.45	2.97	1.11

[a]Amount: % of the original amount of NH_4Cl (1.22 $g.l^{-1}$ = 100%, default SFM_R medium);
q_{Fru} – specific rate of fructose consumption;
q_{SO4} – specific rate of sulfate consumption;
q_{CO2} – specific rate of carbon dioxide production;
CR – carbon recovery (percentage of supplied carbon that is used in the cells constitution);
CCE – carbon conversion efficiency;
$Y_{x/s}$ – cell yield (cell mass produced per mole of substrate consumed).

These results show that steady-state chemostat cultures of strain 1B (SS#1 to SS#4 cultures) were obtained at a dilution rate comprised in the range of 0.07 – 0.08 h^{-1}. Comparing the steady-state continuous cultures, it can be observed that the marked decrease in the metabolic parameters overall, associated to cell growth (OD_{600nm}, biomass, biomass production rate and fructose consumption rate) may be linked with the reduction of the N-source amount, from 100% N to 25% N. The restriction of bacterial growth by the N-source limitation is consistent with the accumulation of C-source that is observed for the formulations with ≤75% N, as 8.15 $g.l^{-1}$ fructose were accumulated by the SS#4

culture (25% N). Moreover, the highest values for CR, CCE and $Y_{x/s}$ that were obtained by the SS#4 culture also highlight that strain 1B directed all the fructose consumed ($\sim$2 g.l^{-1}) to biomass production in response to the nitrogen limitation.

For the production of cost-efficient biocatalysts (resting cells of strain 1B) it is necessary that all C-source and S-source are consumed, since the accumulation of C-source implies the accumulation of S-source in culture and this effect inhibits the desulfurization ability of the resting cells (personal communication). In this context, despite the slight differences observed in the metabolic responses of SS#1 (100% N) and SS#2 (75% N) cultures, only the SS#1 culture had effective cells capable of desulfurizing the DBT or fossil fuels, with fully consumed carbon and sulfur sources. Therefore >75% N is required for the culture to grow and fully consume the C-source as well as produce the maximum effective biocatalysts yield. These results are in agreement with those previously obtained in the N-source minimization shake-flask set of assays (see **Figures 9 and 10**), which have also pointed out for 75% < N-source amount $\leq$ 100% as the optimal amount.

Additionally, flow cytometry was also used to evaluate the influence of N-source amount reduction on the physiological state of the strain 1B cells in each steady-state culture (F#1 to F#4). For flow cytometry analysis, cells were stained with PI and CFDA in order to access membrane integrity and metabolic activity. **Figure 18** presents the dispersion graphics obtained for each steady-state culture analyzed, showing the populations of cells with different physiological states: healthy, stressed (compromised membrane), inactive and dead cells.
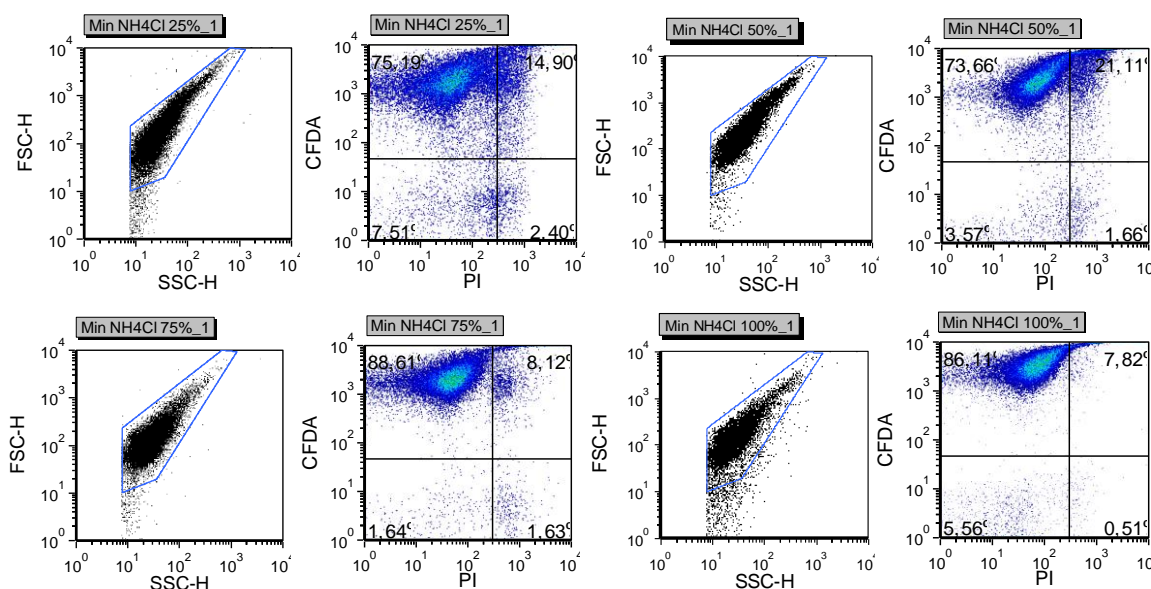
Figure 18 – *Flow cytometric analysis of strain 1B cells from the steady-state cultures, grown in chemostat in SFM$_R$ medium formulations with different N-source (NH$_4$Cl) concentrations: 25%, 50%, 75%, and 100%. In the quadrant analysis, UpL (Upper left quadrant) are healthy cells; UpR (Upper right quadrant) are cells whose membrane is compromised but are metabolically active; LwL (Lower left quadrant) are cells whose membrane is uncompromised but have no metabolic activity; and LwR (Lower right quadrant) are dead cells. Results were obtained in duplicate and each analyzed 6 times.*

Additionally, **Figure 19** shows the bacterial cells viability in each steady-state culture, quantified as percentages of healthy, stressed, inactive and dead cells. These results show the physiological response of strain 1B to the N-source amount reduction in culture medium, demonstrating that the lack of N induced the decrease of healthy cells and consequently the increase of stressed, inactive and/or dead cells. In SS#4 culture (25% N) it can be observed the highest percentage of inactive (7.6%) and dead cells (2.7%), as well as a high value of stressed cells (14.7%). In SS#3 culture (50% N), the lack of N-source is translated mainly by a great increase of stressed cells, from 7.5% (SS#1 – 100% N) to 21.8%, and consequently the decrease of healthy cells, from 82.5% (SS#1) to 71%.
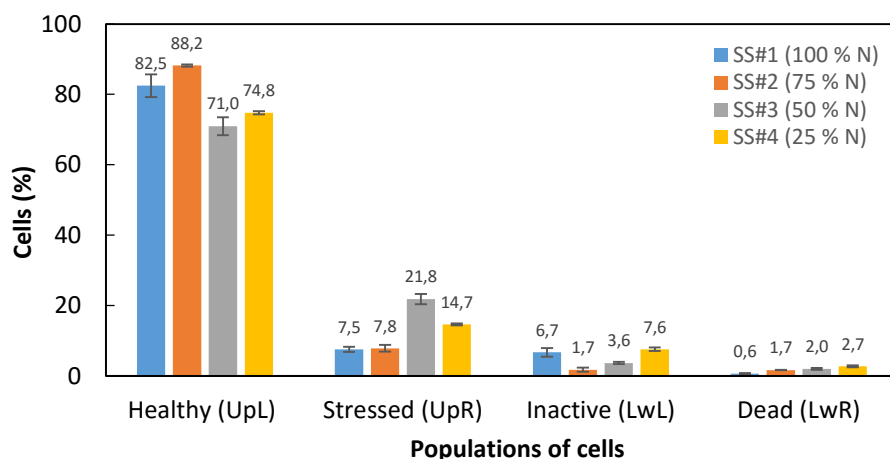

Figure 19 – *Bacterial cells viability (as percentages of healthy, stressed (compromised membrane), metabolically inactive, and dead cells) in each one of the four steady-state cultures, from different N-source (NH_4Cl) amounts: 25%, 50%, 75%, and 100%. UpL, UpR, LwL, and LwR are the quadrants designation (see figure 10). Standard deviation (n = 12) is indicated by the error bars.*

Moreover, these results also indicate that SS#1 and SS#2 cultures (100% N and 75% N) have a comparable percentage of healthy cells, 82.5% and 88.2%, respectively. The slight difference observed can be associated to the fact that the SS#1 culture consumed the C-source available in the medium causing the beginning of a starvation period, in contrast with what happened in SS#2 culture, which accumulated C-source and S-source and therefore the C-source was not limiting. However, SS#2 culture, despite presenting the highest percentage of viable cells (94%, healthy + stressed cells), was not able of further DBT desulfurization due to the S-source accumulation in the culture medium, as already referred.

Since the optimal N-source amount for *G. alkanivorans* strain 1B to grow and fully consume the C-source would be above 75% and lower than 100%, an interpolation concerning this amount was carried out based on a polynomial regression fit to BPR = f(N-source amount), where BPR is the biomass production rate, a parameter indicator of the bacterial growth (**Figure 20**).
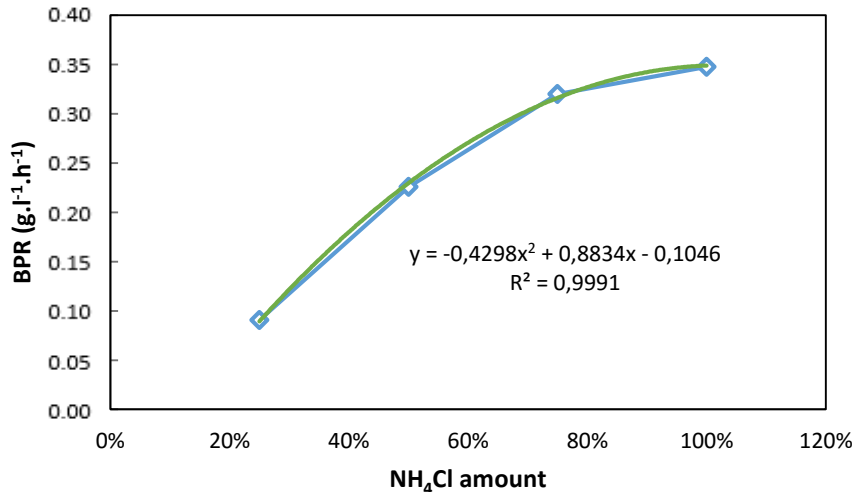
Figure 20 – Polynomial regression for the biomass production rate (BPR) versus N-source amount.

Based on this model, the minimal N-source amount stipulated to apply in further minimum culture medium optimization assays in chemostat was 90%, since the BPR estimated is similar to that of 100% (0.34 g.l^{-1}.h^{-1} for 90% N and 0.35 g.l^{-1}.h^{-1} for 100% N).

3.2.2. Minimized medium: commercial versus alternative C-source

Based on the minimal amounts stipulated for N-source (90%), Mg-source (25%) and TES (25%) from the prior set of assays (shake-flask and low-cost chemostat), two more continuous cultures (SS#5 and SS#6), were carried out towards the development of the optimal minimized medium for bioreactor (SFMM$_R$ – SFM$_R$ minimum). This process aimed to achieve the maximum yield of effective biocatalysts (strain 1B resting cells).

As already referred, in chemostat, the microbial specific growth rate can be optimal if the dilution rate and culture conditions are well defined. Therefore, for faster growth and maximum biomass yield, the highest dilution rate possible should be used. However, if a dilution rate is too high there will be accumulation of the culture medium components, leading to nutrient surplus and, in the limit, to cell washout.

The dilution rates for the two SFMM$_R$ medium continuous cultures (using different C-sources, Fru+Glu *versus* JAJ$_c$ (SS#5 *vs* SS#6, **Table 3**)) were chosen based in the average growth rates obtained in the correspondent SFMM medium for shake-flask assays (F#14: μ = 0.07 and F#15: μ = 0.05).This choice was based on the fact that the μ$_{max}$ (0.08 h^{-1} and 0.06 h^{-1}, **Table 6**) could be too high causing nutrient accumulation, namely sulfate. Sulfate accumulation will further inhibit the desulfurization ability of the produced biocatalysts, hindering their potential application for BDS processes.

Additionally, due to the possibility of JAJ$_p$ causing further inhibition of BDS probably due to the residual BaCl$_2$ present in the juice, as referred for F#1' and F#15 batch cultures (shake-flask assays), a

different JAJ was used as C-source: JAJ_c (see **section 2.2** for details). This JAJ_c has a higher ratio of carbon/sulfate (300 g.l^{-1} total sugars/ 600 mg.l^{-1} sulfate), enabling the use of the juice without $BaCl_2$ precipitation. Since its dilution to 10 g.l^{-1} total sugars in the culture medium also account for the optimal sulfate amount without requiring additional S-source (see F#6, **Table 3**).

The metabolic characteristics for both bacterial culture steady-states, in the minimized medium ($SFMM_R$ medium, see F#5 and F#6 in **Table 3**), using Fru+Glu (SS#5) *versus* JAJ_c (SS#6) are registered in **Table 8**. Operational conditions were maintained constant; therefore the culture behavior translates the physiological response of the microbial cells to the influence of the C-source in the minimized culture medium ($SFMM_R$ medium), with 90% N, 25% Mg and 25% TES.

Comparing the two steady-state cultures, SS#5 *versus* SS#6, it can be observed that SS#5 culture presented a higher biomass production (3.97 g.l^{-1}) than SS#6 (3.10 g.l^{-1}), which is consistent with the overall metabolic parameters associated to growth (OD_{600nm}, biomass, biomass production rate, sugars consumption rates, q_{Fru}, q_{Glu}). Moreover, the biomass production rate of SS#5 was 1.7-fold higher than the one obtained with SS#6, which could be explained by the higher dilution rate used (0.065 h^{-1} *versus* 0.047 h^{-1}). The results obtained for CR, CCE and $Y_{x/s}$ are very similar for both SS cultures, demonstrating an overall similar behavior of the cells grown in the minimized medium, despite the different C-source. In addition, the $SFMM_R$ medium also permitted the total consumption of the available sugars in both SS cultures, avoiding any nutrient accumulation which may be a BDS inhibitor.

Table 8 – *Metabolic parameters for the steady-state cultures of <u>G. alkanivorans</u> strain 1B, grown in chemostat, in the minimized medium ($SFMM_R$ medium) using Fru+Glu (SS#5) or JAJ_c (SS#6) as C-source.*

Minimized culture medium ($SFMM_R$)[a]	Glu+Fru (SS#5)	JAJ_c (SS#6)
Dilution rate (h[-1])	0.065	0.047
Biomass production rate (g.l^{-1}.h[-1])	0.26	0.15
Fructose Consumption rate (g.l^{-1}.h[-1])	0.52	0.28
Glucose Consumption rate (g.l^{-1}.h[-1])	0.13	0.11
q_{Fru} (mmol.g^{-1} (DCW).h[-1])	0.73	0.50
q_{Glu} (mmol.g^{-1} (DCW).h[-1])	0.18	0.20
q_{SO4} (mmol.g^{-1} (DCW).h[-1])	3.13	2.54
q_{CO2} (mmol.g^{-1} (DCW).h[-1])	1.72	1.66
CR (%)	80.90	86.16
CCE (%)	49.70	46.80
$Y_{x/s}$ (g (DCW).mol^{-1})	71.46	67.30
Accumulated carbon source (g.l^{-1})	0.00	0.00
OD_{600nm}	11.29	10.03
Biomass (g.l^{-1})	3.97	3.10
q_{2-HBP} (µmol.g[-1](DCW).h[-1])	2.90	12.23

[a]SFM_R minimum medium with 90% N, 25% Mg and 25% TES

Note: see table 7 endnotes for details on the different parameters

Further desulfurization results obtained with resting cells from SS#5 ($q_{2\text{-HBP}}$ = 2.90 µmol.g^{-1}(DCW).h^{-1}) and SS#6 ($q_{2\text{-HBP}}$ = 12.23 µmol.g^{-1}(DCW).h^{-1}) showed a 4-fold higher desulfurization ability from the biocatalysts produced in the SFMM$_R$ medium using the alternative C-source (JAJ$_c$).

Additionally to the metabolic study, flow cytometry analysis (**Figure 21**) was also performed to evaluate the physiological state of the cells from each SS culture (SS#5 and SS#6). Flow cytometry results show a percentage of viable cells >93% for both cultures (SS#5: ~95% = 62% healthy + 33% stressed; SS#6: ~93% = 73% healthy + 20% stressed) and consequently <7% of inactive and dead cells (SS#5: 3.6% inactive + 1.1% dead; SS#6: 3.7% inactive + 2.8% dead), demonstrating the overall good physiological state of each culture.
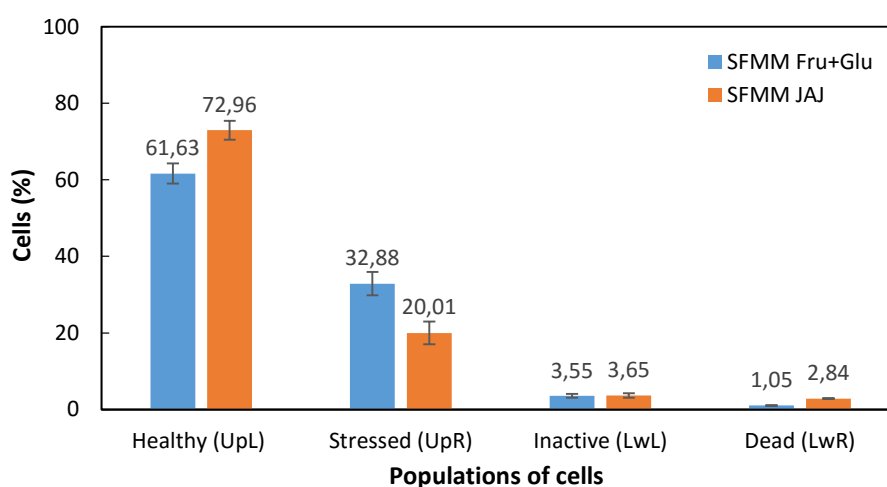

Figure 21 – Bacterial cells viability (as percentages of healthy, stressed (compromised membrane), metabolically inactive, and dead cells) in both steady-state cultures, grown in SFMM$_R$ medium with Glu+Fru (SS#5) and JAJ$_c$ (SS#6). UpL, UpR, LwL, and LwR are the quadrants designation (see figure 18).
Standard deviation (n = 12) is indicated by the error bars.

Therefore, the overall metabolic and physiological results pointed out for SS#6 culture resting cells as the optimized cost-effective biocatalysts for BDS processes with *G. alkanivorans* strain 1B. In fact, these efficient biocatalysts are capable of enhanced DBT desulfurization ($q_{2\text{-HBP}}$ = 12.23 µmol.g^{-1}(DCW).h^{-1}) and may be produced in a chemostat using a low-cost culture medium: SFMM$_R$ + JAJ$_c$ culture medium. DBT desulfurization using strain 1B SS#6 resting cells (JAJ$_c$) was 2.4-fold higher than that obtained by Silva (2012) using strain 1B growing cells and JAJ$_p$ as C-source ($q_{2\text{-HBP}}$ = 5.06 µmol.g^{-1}(DCW).h^{-1}).

3.3. Evaluation of the expression of the *dsz* operon by Real Time-PCR

In complement to the BDS assays using resting cells of SS#5 and SS#6 cultures, the evaluation of the desulfurization gene expression (regulated by *dsz* operon) in both chemostat cultures was performed through a Real Time-PCR system (RT-PCR). A two-step RT-PCR approach was performed as described in **section 2.6.6**. For each SS culture, the RNA was first extracted from cells

and then retro-transcribed into cDNA and amplified with primers for *dsz*A, *dsz*B, *dsz*C and *16s*, the latter being used as a control.

A comparative study of the gene expression results for cells of SS#5 culture (SFMM$_R$ medium, Fru+Glu) *versus* cells of SS#6 culture (SFMM$_R$ medium, JAJ$_c$) showed that the overall gene expression for the three *dsz* genes was higher for SS#6 culture cells, despite of the different levels of expression by each gene: *dsz*C expression was 22% lower for SS#5 cells (Ratio SS#5/SS#6 = 0.78), *dsz*A was 83% lower for SS#5 cells (Ratio SS#5/SS#6 = 0.17) and *dsz*B was 99% lower for SS#5 cells (ratio SS#5/SS#6 = 0.01). These results are in accordance with the higher desulfurization achieved by SS#6 biocatalysts ($q_{2\text{-HBP}}$ = 12.23 µmol.g^{-1} (DCW).h^{-1}).

3.4. Desulfurization of a model oil

In order to scale-up the BDS process with resting cells of *G. alkanivorans* strain 1B into a petroleum refinery it is necessary to first evaluate their potential to desulfurize crude oils, which are complex mixtures rich in thiophenic compounds that are recalcitrant to HDS. Crude oil BDS has several associated problems that must be taken into account, such as phase separation between the water and organic phase; toxicity of the organic solvent or inhibition of desulfurization due to the presence of multiple substrates.

Crude oil was simulated through the design of a model oil, consisting on a mixture of DBT (dibenzothiophene) and several DBT derivatives dissolved in o-xylene, and posteriorly prepared and tested in a BDS assay using resting cells of SS#6 culture in a biphasic system (organic phase: model oil + aqueous phase: resting cells re-suspended in a phosphate solution).

Figure 22 shows the gas chromatography (GC) profiles for the model oil desulfurization by strain 1B SS#6 resting cells. This figure shows that all DBT derivatives in the mixture were reduced to the respective product: 2-HBP for DBT, 4-methyl-2-HBP for 4-methyl-DBT, 4,6-dimethyl-2-HBP for 4,6-dimethyl-DBT and 4,6-diethyl-2-HBP for 4,6-diethyl-DBT.

Therefore, a complete desulfurization of the model oil by strain 1B was observed within 72 hours. The ability of *G. alkanivorans* strain 1B to desulfurize a model oil, with DBT, 4-methyl-DBT and 4,6-dimethyl-DBT, was already described by Alves *et al.* (2008). Based on each $q_{2\text{-HBP}}$ value obtained during the mixture desulfurization, these researchers also demonstrated that strain 1B has a selective preference for: DBT < 4-methyl-DBT < 4,6-dimethyl-DBT.

These results show the potential of strain 1B to desulfurize complex mixtures of DBT derivatives. Nevertheless, much improvement must be still carried out towards its possible industrial scale-up, as a complement to HDS into a crude oil refinery.
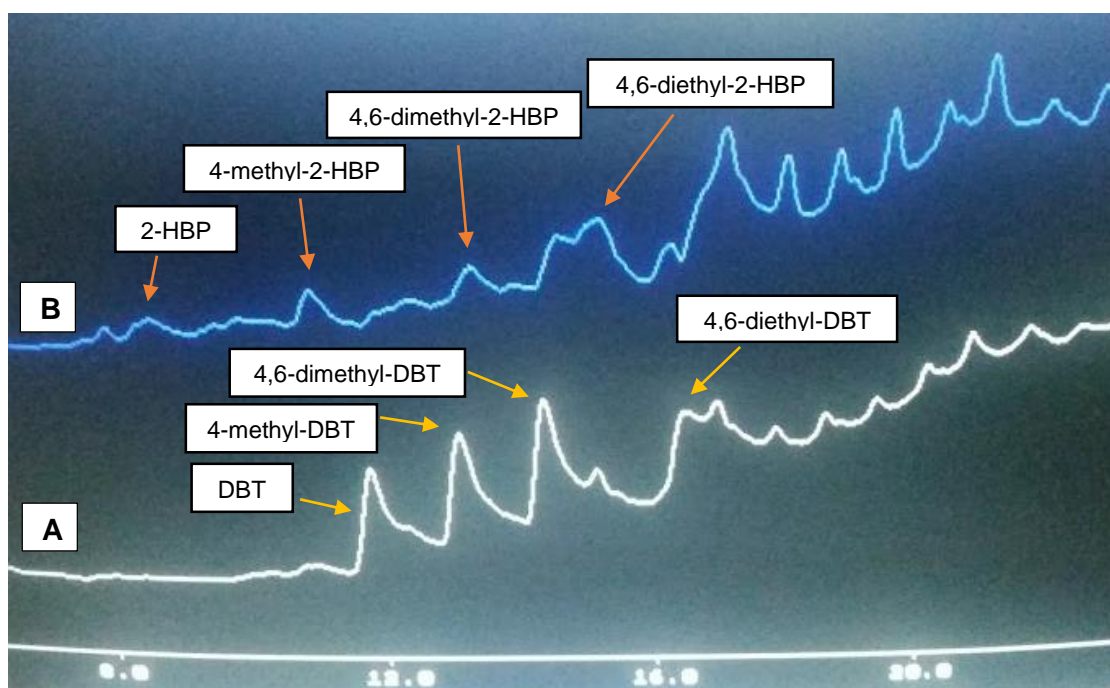
Figure 22 – Gas chromatograms from the model oil desulfurization by SS#6 resting cells. A – Model oil components before desulfurization; B – Model oil components after 72 hours of desulfurization.

3.5. Pigments analysis of pre and post-desulfurizing resting cells

One of the strategies used to make industrial bioprocesses economically viable is to take advantage of high added value products resulting from the biomass (Makkar & Cameotra 1999; FitzPatrick *et al.* 2010; Veiga-Crespo *et al.* 2012). One example of such products are carotenoids, pigments that are produced by many microorganisms and have a high market value.

To evaluate if secondary metabolites, such as pigments, produced by *G. alkanivorans* strain 1B could be affected by the desulfurization process, pigments extraction of samples from pre- and post-desulfurization assays (Pre BDS, Post BDS) using both SS#5 and SS#6 resting cells were performed. **Figure 23 (A-D)** shows a picture of the dried biomass of each sample extracted for pigments quantification. All biomass samples presented an orange color, however, there is a visible difference between the color of SS#6 resting cells biomass before (**Figure 23C**) and after desulfurization (**Figure 23D**), presenting the latter a more intense orange.
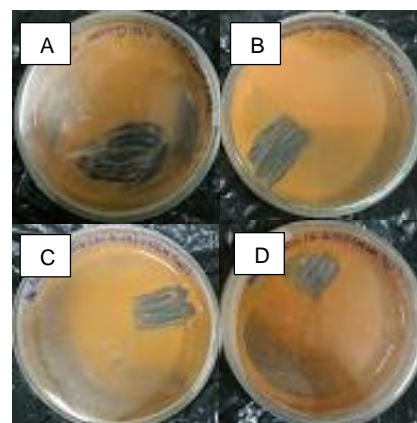
Figure 23 – Samples of dried biomass from the pre and post-BDS assays using SS#5 and SS#6 resting cells. A – SS#5-PreBDS; B – SS#5-PostBDS; C – SS#6-PreBDS; D – SS#6-PostBDS.

Figure 24 presents the results of the pigments analysis by HPLC of the four extracts obtained. The analysis of the chromatograms revealed the presence of several carotenoid related peaks, of which only lutein, astaxanthin and canthaxanthin were identifiable, representing less than 50% of the total.

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Overall, the SS#5-preBDS cells (**Figure 23A**), from SFMM$_R$+ (Fru+Glu) medium, were able to produce 116 μg.g^{-1}(DCW) of lutein and 18 μg.g^{-1}(DCW) of astaxanthin. No canthaxanthin was observed. For SS#5-postBDS cells (**Figure 23B**), it was observed an increased production of lutein and astaxanthin, 189 μg.g^{-1}(DCW) and 49 μg.g^{-1}(DCW), respectively. In this case, canthaxanthin was also produced, attaining 27 μg.g^{-1}(DCW). Relatively to the cells grown in SFMM$_R$ + JAJ$_c$ medium, for SS#6-preBDS cells it was observed a production of 100 μg.g^{-1}(DCW) of lutein, 13 μg.g^{-1}(DCW) of astaxanthin and 8 μg.g^{-1}(DCW) of canthaxanthin. Again, an increase of the overall pigments production was observed for the SS#6-postBDS cells, attaining 184 μg.g^{-1}(DCW) of lutein, 114 μg.g^{-1}(DCW) of astaxanthin and 36 μg.g^{-1}(DCW) of canthaxanthin. From these results, it can be stated that the exhausted resting cells (SS#5-postBDS; SS#6-postBDS) produced higher amounts of all identified carotenoids (lutein, astaxanthin and canthaxanthin) than fresh resting cells (SS#5-preBDS; SS#6-postBDS). Lutein was the most abundant carotenoid, both for pre- and post-desulfurization resting cells from SS#5 and SS#6 cultures. Astaxanthin and canthaxanthin are pigments with orange and red color, respectively. The presence and increase of these pigments justify the difference in color observed in the petri dishes (**Figure 23**).
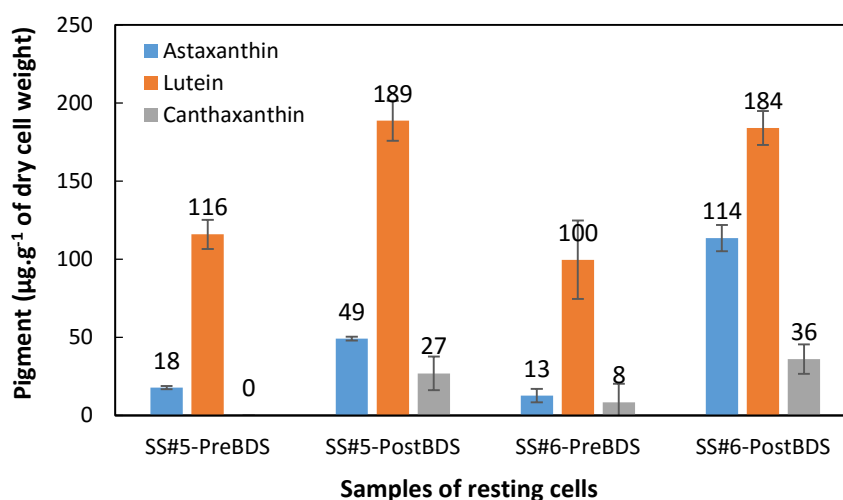

Figure 24 – *Analysis of the main carotenoids produced by pre- and post-desulfurization strain 1B cells from SS#5 and SS#6 resting cells (RC). SS#5-preBDS and SS#5-postBDS are resting cells produced in the chemostat with SFMM$_R$ + (Fru+Glu) medium; SS#6-preBDS and SS#6-postBDS are resting cells produced in the chemostat with SFMM$_R$ + (JAJ$_c$) medium. PreBDS –pre-desulfurization (fresh RC); PostBDS – post-desulfurization (exhausted RC).*

These results also show a different profile on carotenoids production for resting cells originated in a minimized medium with Fru+Glu (SS#5) *versus* JAJ$_c$ (SS#6), as C-source. SS#5-postBDS attained a total pigments production of about 265 μg.g^{-1}(DCW) in comparison to the 334 μg.g^{-1}(DCW) for SS#6-postBDS cells. The total carotenoids produced by *G. alkanivorans* strain 1B cells was higher than those obtained by *G. jacobaea* (CECT 5282), 227 μg.gDCW^{-1}, a genetically modified *Gordonia* strain used for pigment production (De Miguel *et al.* 2000; De Miguel *et al.* 2001; Veiga-Crespo *et al.* 2005; Veiga-Crespo *et al.* 2012). This brings good prospectives to the optimization of pigments production towards their utilization in the agro-alimentary industry.

Since DBT desulfurization enhanced the overall pigments production in the resting cells used as biocatalysts, the further exploitation of these high added value products from the exhausted microbial biomass can be coupled to BDS process as a decisive factor to bring economical balance to its possible scale-up (Paixão *et al.* 2016).

4. Conclusion

The shake-flask minimization assays allowed the design of a minimized culture medium (SFMM medium) containing 75-100% of N-source, 25% of Mg-source and 25% of TES (% of the original amounts used in the default SFM medium). Using 85% N, the SFMM medium was tested with two C-sources (Fru+Glu) *versus* JAJ_p and the results obtained demonstrate that independently of the C-source used, the SFMM medium enhances the desulfurization by strain 1B in comparison with the respective control (SFM medium).

Based on these minimal amounts established for SFMM medium, the next step was to apply this combined reduction in the SFM_R medium and optimize the culture conditions in chemostat assays, where bacteria can be grown in a physiological steady-state under constant and optimal conditions. In this context, after a first screening on the minimal amount for N-source using a low-cost bioreactor, the established $SFMM_R$ medium (90% N, 25% Mg and 25% TES) was tested in two more continuous cultures (SS#5 and SS#6) for the development of the optimal minimized medium for reactor taking into account the maximum yield of effective biocatalysts (*G. alkanivorans* strain 1B resting cells). The results highlighted the $SFMM_R$ medium with JAJ_c as C-source as the optimal minimized medium in which cost-effective biocatalysts can be produced (SS#6 resting cells), attaining a desulfurization (q_{2-HBP}) of 12.2 $\mu mol.g^{-1}(DCW).h^{-1}$, about 4-fold higher than the one observed with the biocatalysts from the $SFMM_R$ medium with Fru+Glu as C-source (q_{2-HBP} = 2.9 $\mu mol.g^{-1}(DCW).h^{-1}$ for SS#5 resting cells). RT-PCR results confirmed the higher expression of the overall desulfurization genes (*dsz*A*, dsz*B*, dsz*C genes) in the SS#6 resting cells.

Moreover, a BDS assay for a model oil (500 μM, DBT + 4-methyl-DBT + 4,6-dimethyl-DBT, and 4,6-diethyl-DBT), using SS#6 resting cells culture in a biphasic system, was performed showing the ability of complete desulfurization within 72 h by these biocatalysts. Additionally, it was also demonstrated that the exhausted biocatalysts were able to produce high amounts of carotenoids (334 $\mu g.g^{-1}(DCW)$). The further exploitation of these high added value products from the exhausted microbial biomass can also be coupled to the BDS process as a decisive factor to bring economical balance to its industrial scale-up in addition to the low-cost culture medium ($SFMM_R$ medium) developed for the effective biocatalysts production in bioreactor. This reflects great perspectives for the future of BDS as an integrated part of the petroleum treatment industry.

5. Future Works

As a future work, it is important to further test the influence of magnesium in desulfurization by strain 1B. It is also important to test desulfurizing cells with crude oil or diesel/gasoline, in order to better understand how *G. alkanivorans* strain 1B cells react in "real" fossil fuels. Finally, more tests must be conducted to optimize conditions to maximize both desulfurization and pigment production during desulfurization.

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