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Programa de Pós-Graduação em Biotecnologia

Estudo da atividade microbiológica associada à glicerina como doador de elétrons e substrato em processos de redução microbiana

Sidnei Cerqueira dos Santos

Salvador-BA
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Estudo da atividade microbiológica associada à glicerina como doador de elétrons e substrato em processos de redução microbiana

Defesa de tese apresentado ao Programa de Pós-Graduação em Biotecnologia, da Rede Nordeste de Biotecnologia (RENORBIO), ponto focal Universidade Federal da Bahia (UFBA), como requisito para obtenção do título de Doutor em Biotecnologia.

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ATA – DEFESA DE TESE

Ata de Defesa de Tese de Doutorado do aluno **SIDNEI CERQUEIRA DOS SANTOS.** Aos dezenove dias do mês de junho do ano de dois mil e treze, às 09:00 horas, reuniu-se a banca de Defesa de Tese composta pelos Professores Doutores Cristina Maria Quintella, Universidade Federal da Bahia, Presidente, Rogéria Comastri de Castro Almeida, Universidade Federal da Bahia, Juan Carlos Rossi Alva, Universidade Católica de Salvador, Milton Ricardo de Abreu Roque, Universidade Federal da Bahia, Iracema Andrade Nascimento, Universidade Federal da Bahia, perante o qual **SIDNEI CERQUEIRA DOS SANTOS,** aluno regularmente matriculado no Curso de Doutorado em Biotecnologia da Rede Nordeste de Biotecnologia – RENORBIO, Ponto Focal Bahia, defendeu, para preenchimento do requisito de doutor, sua Tese intitulada **"Estudo da atividade microbiológica associada à glicerina como doador de eletros e substrato em processos de redução microbiana".** A defesa da referida tese ocorreu, das 09:00 horas às 12 e 30 horas, tendo o doutorando sido submetido à sabatina, dispondo cada membro da banca do tempo para tal. Finalmente, a banca reuniu-se em separado e concluiu por considerar o doutorando aprovado por sua tese e sua defesa terem, por unanimidade, recebido o conceito satisfatório.

Eu, Cristina Maria Quintella, que presidi a Banca de Tese, assino a presente Ata, juntamente com os demais membros e dou fé. Em Salvador, 19 de junho de 2013.

Profa. Dra. Cristina Maria Quintella – UFBA
(Orientadora)

Prof. Dr. Juan Carlos Rossi Alva - UCSAL
(Examinador)

Profª. Drª. Rogéria Comastri de Castro Almeida - UFBA
(Examinadora)

Profa. Dra. Iracema Andrade Nascimento - UFBA
(Examinadora)

Prof. Dr. Milton Ricardo de Abreu Roque - UFBA
(Examinador)

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Muito obrigado a todos!

RESUMO

A glicerina bruta (GB), co-produto da produção do biodiesel, é uma interessante fonte de glicerol. A conversão microbiana de glicerol em processos de redução biológica de sulfato pode ser uma alternativa promissora para o aproveitamento e valorização deste subproduto. Neste sentido, o objetivo do trabalho foi estudar processos de redução microbiana associados ao uso de glicerina bruta e glicerina comercial como substratomostras de GB derivadas de mamona, soja, algodão e óleos e gorduras residuais (OGR) foram utilizadas como fonte de carbono e energia. As bactérias redutoras de sulfato (BRS) utilizadas nas pesquisas tiveram origem de ambientes anóxicos. Os experimentos foram monitorados através de testes microbiológicos, químicos, bioquímicos e moleculares. Os resultados mostraram que a GB é uma interessante, de baixo custo e abundante fonte de carbono em processo de redução microbiana. Entretanto, novos estudos devem ser realizados para compreender os mecanismos da GB em altas concentrações na formação biológica de sulfeto. Os resultados também demonstraram que a glicerina comercial estimulou as BRS em diferentes ambientes e que o mesmo pode ter aplicação potencial em processos de precipitação de metais.

Palavras-Chave: Glicerina Bruta, Glicerina Comercial, Bactérias Redutoras de Sulfato, Bactérias fermentativas

ABSTRACT

Crude glycerol (CG), co-product of biodiesel production, is an interesting source of glycerol. Microbial conversion of glycerol in biological reduction processes of sulfate can be a promising alternative for the use and to improve the economic value of this byproduct. In this work, the objective was to study biological reduction processes associated to use of crude glycerol and glycerin as substrate. Samples of CG derivative castor beans, soybeans, cotton and oils and waste fats, and glycerin were used as source carbon and energy. The sulfate-reducing bacteria (SRB) used in the research were from anoxic environment. The experiments were monitored by microbiological, chemistry, biochemistry and molecular tests. The results showed that the CG showed to be an interesting, cheap and abundant source carbon in microbial reduction processes. However, new studies have to be performed to understand the mechanisms of CG in high concentration on biological sulfide formation. The results also showed that glycerin stimulated BRS in different environments and it can be potential application in metals precipitation processes.

Keywords: Crude Glycerol, Glycerin, Sulphate-Reducing Bacteria, Fermentative Bacteria

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LISTA DE ABREVIATURAS E SIGLAS

AFS – Adenosina fosfosulfato

AMP – Adenosina monofosfato

AP – Água de Produção

APB55 – Cultura mista de bactérias redutoras de sulfato

ATP – Adenosina trifosfato

BA – Bahia

BLST – Local Alignment Search Tool

BRS – Bactérias redutoras de sulfato

CC – Compostos Carbonílicos

Cd – Cá dmio

Cl – Cloro

CMI – corrosão microbiologicamente induzida

Cr – Cromo

DGGE – Denaturating gradient gel electrophoresis

CG – Crude glyceriol

2,4-DNPH – 2,4-dinitrofenilhidrazina

2,4-DNPHo – 2,4-dinitrofenilhidrazina

FAFS – Fosfoadenosina fosfosulfato

FAFS – Fosfoadenosina fosfosulfato

Fe – Ferro

GB – Glicerina bruta

Hg – Mercúrio

K – Potássio

KOH – Hidróxido de potássio

MEOR – recuperação avançada de petróleo com micro-organismos

Mn – Manganês

Na – Sódio

NaOH – Hidróxido de sódio

NCBI – National Center for Biotechnology Information

Ni - Níquel

OD – Optical density

OGR – Óleos e gorduras residuais

Pb – Chumbo

PCR – Reação em Cadeia da Polimerase

PPi – Pirofosfato inorgânico

1,3-PDO – 1,3-propanediol

Ra – Rádio

SRB – Sulfate-reducing bacteria

TR – Tinto river

U – Urânio

Zn – Zinco

SUMÁRIO

Introdução

As fontes não renováveis de energia como, por exemplo, os combustíveis fósseis, são a mais utilizada no mundo. Esse quadro de predomínio dos combustíveis fósseis na matriz energética mundial fez surgir duas preocupações principais: as questões relacionadas à segurança energética, porque a velocidade de geração deste pela Natureza é inferior à velocidade com que é utilizado para gerar energia, o que pode levar ao seu esgotamento; e a mitigação das mudanças climáticas, devido à liberação para a atmosfera terrestre de grande quantidade de dióxido de carbono e outros gases tóxicos. Nesse contexto, a implantação de fontes renováveis de energia é essencial. Energia renovável é aquela originária de fontes naturais que possuem a capacidade de renovação, tais como energia eólica, energia hidráulica, biomassa. As energias renováveis são capazes de prover maior segurança energética e o seu aproveitamento em maior escala é um dos principais instrumentos de combate às mudanças climáticas decorrentes da elevação dos gases de efeito estufa na atmosfera. Além disso, essas formas de energia podem também contribuir para o desenvolvimento social e econômico, para a universalização do acesso à energia e para a redução de efeitos nocivos ao meio ambiente e à saúde (IPCC, 2011; BRASIL, 2013).

O biodiesel é introduzido na matriz energética brasileira e atende a um dos interesses prioritários do Governo Federal (Lei 11.097 de 13 de janeiro de 2005), no uso de fontes de energias renováveis. A inclusão do biodiesel na matriz energética tem sido destaque em vários países e blocos comerciais. O aumento na demanda do biodiesel se deve, principalmente, a vantagens como os benefícios ao meio ambiente, proporcionado pela redução das emissões e a reutilização do CO_2 pelos vegetais utilizados como matéria-prima, bem como a sua biodegradabilidade (QUINTELLA et al., 2009). O biodiesel é uma fonte de energia limpa, não poluente e que pode ser utilizada pura ou misturada com o diesel derivado do petróleo. A utilização do biodiesel em um motor diesel convencional proporciona, quando comparado com a queima do diesel, uma redução das emissões de gases poluentes (VYAS; VERMA; SUBRAHMANYAM, 2010). Desde o início de 2010 todo abastecimento feito com óleo diesel tem o novo percentual de 5% de biodiesel e a expectativa é de atingir 20% do biocombustível até 2015 (BIODIESEL.GOV.BR). O biodiesel é produzido a partir de óleos vegetais, obtidos da soja, mamona, dendê, milho, girassol, babaçu, palma, algodão, e gorduras animais, por meio da transesterificação com etanol ou metanol (alcoólise), catalisada por

NaOH ou KOH, e gera como subproduto a glicerina bruta (FERRARI et al., 2005; MU et al., 2006).

A glicerina bruta (GB) é o principal subproduto da produção do biodiesel (FERRARI et al., 2005; MU et al., 2006). A produção de 100 kg de biodiesel apresenta um rendimento de aproximadamente 10 kg de GB, com cerca de 40-90% de glicerol (HAZIMAH; OOI; SALMIAH, 2003; QUINTELLA; CASTRO, 2009). A glicerina derivada da produção do biodiesel possui baixo valor agregado, pois contém resíduos de metanol, hidróxido de sódio, ácidos graxos livres, sais de ácido graxo, ésteres, compostos de enxofre, proteínas e minerais, que torna esse subproduto inadequado para uso direto na indústria e a sua purificação tem um custo mais elevado do que a obtenção da glicerina por outras fontes (OOI et al., 2004; QUINTELLA et al., 2006; THOMPSON; HE, 2006). Este cenário indica que a viabilização comercial do biodiesel está condicionada ao consumo deste volume extra de glicerina bruta (MOTA; SILVA; GONÇALVES, 2009), e nesse sentido, novas e sustentáveis estratégias baseadas na conversão microbiana da GB por processos biotecnológicos em produtos de valor agregado estão sendo propostas (PYLE; GARCIA; WEN, 2008; YAZDANI; GONZALES, 2007), como, por exemplo, a conversão microbiana de glicerol a 1,3-propanodiol usado na produção de poliésteres (ITO et al., 2005). O glicerol é um substrato com alta capacidade de redução. Os micro-organismos são capazes de assimilar o glicerol como fonte de carbono para obtenção de energia metabólica, uma vez que este é frenquentemente formado como intermediário no catabolismo de lipídios e glicose, tanto em condições aeróbicas quanto anaeróbicas (ASHBY et al., 2005; PAPANIKOLAOU et al., 2002).

As bactérias redutoras de sulfato (BRS) são micro-organismos anaeróbios amplamente distribuídos em ambientes anóxicos, onde eles usam sulfato como aceptor de elétrons terminal para a degradação de compostos orgânicos, resultando na produção de sulfeto (MUYZER; STAMS, 2008; POSTGATE, 1984). As BRS são importantes membros de comunidades microbianas de interesse econômico, ambiental e biotecnológico (MARTINS et al., 2009). O sulfeto, tóxico e corrosivo, gerado pelas BRS causa uma série de problemas para indústria petrolífera. Por outro lado, a redução biológica de sulfato tem aplicações positivas para biorremediação de áreas impactadas com metais pesados, porque as BRS possuem a capacidade de formar precipitados insolúveis, através da reação do sulfeto com metais pesados dissolvidos (BENEDETTO et al., 2005). A redução de sulfato pelas BRS tem sido indicada para o tratamento de uma variedade de efluentes industriais contaminados com sulfato (ICGEN, 2006; GIBERT et al., 2004), sendo a drenagem ácida de minas a mais referenciada, por ser um dos mais graves impactos ambientais associados à atividade de

mineração (LUPTAKOVA; MARIA, 2005; MUYZER; STAMS, 2008). As BRS também podem atuar imobilizando metais pesados tóxicos, como cromo e urânio, reduzindo estes em formas menos móveis (WALL; KRUMHOLZ, 2006).

Dessa forma, a glicerina bruta, além de ser de baixo custo e abundante, é também um interessante fonte de glicerol, que pode ser uma alternativa promissora em processos biotecnológicos, como a redução biológica de sulfato (DHARMADI; MURARKA; GONZALEZ, 2006).

Objetivo Geral

O objetivo do trabalho foi estudar os processos de redução microbiana associados ao uso de glicerina derivado do biodiesel e glicerina comercial, como fonte de carbono e energia, a partir de cultura de bactérias redutoras de sulfato (BRS) provenientes de ambientes anóxicos, visando potencializar a aplicação biotecnológica e contribuir comercialmente com as energias renováveis

Objetivos Específicos

Investigar a presença de BRS em amostras de água produzida.

Selecionar BRS de amostras de água produzida para ensaios de produção de sulfeto.

Avaliar a produção de sulfeto a partir de cultura mista de BRS selecionadas usando glicerina bruta como única fonte de carbono e energia.

Determinar a concentração de compostos carbonílicos nas amostras de glicerina bruta;

Desenvolver uma metodologia com potencial de aplicação biotecnológica;

Avaliar a redução biológica de sulfato a partir de amostras provenientes de sedimentos anóxicos usando glicerol e sulfato em pH ácido e neutro;

Isolar as bactérias oriundas dos enriquecimentos;

Avaliar a produção de sulfato e sulfeto das culturas;

Analisar os metabólitos produzidos a partir da degradação de glicerol;

Identificar e caracterizar os micro-organismos envolvidos.

CAPÍTLULO 1: REVISÃO DE LITERATURA

Bioquímica e Fisiologia de Bactérias Redutoras de Sulfato (BRS)

As bactérias redutoras de sulfato representam um grupo formado por micro-organismos essencialmente quimiorganotróficos e anaeróbios, que realizam a redução de sulfato (SO_4^{2-}) a sulfeto (H_2S, HS^- e S^{-2}). Nesse processo apenas uma pequena parcela do enxofre reduzido é assimilada pelos micro-organismos, chamado de redução assimilativa, onde o sulfato é incorporado como fonte de enxofre para os processos de biossíntese, enquanto que na redução desassimilativa ou dissimilativa o sulfato é utilizado como aceptor final de elétrons para a geração de energia, sendo consequentemente excretado na forma de íon sulfeto, normalmente hidrolisado a H_2S livre (Figura 1) (POSTGATE, 1984).

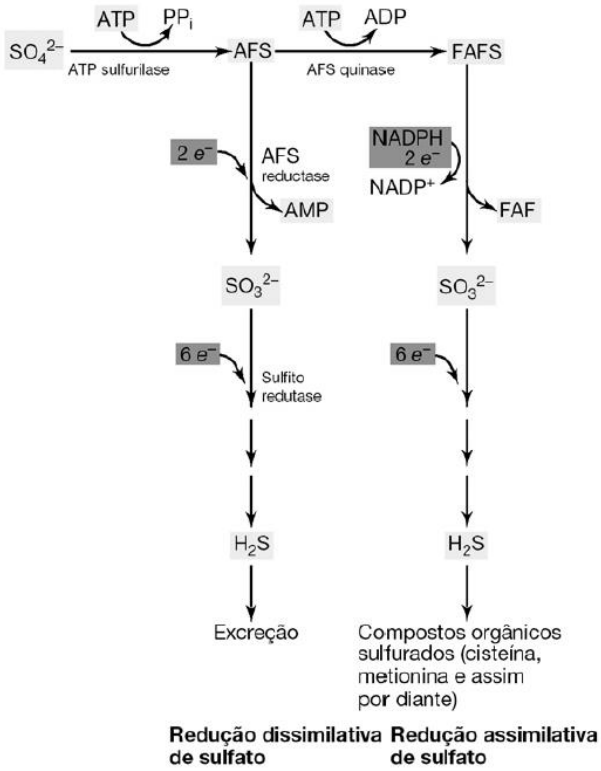

Figura 1. Via metabólica da produção de sulfeto. Esquema das reduções assimilativa e dissimilativa de sulfato: AFS, adenosina fosfosulfato; FAFS, fosfoadenosina fosfosulfato (MADIGAN et al., 2009, modificado).

A redução do sulfato a sulfeto se inicia com a ativação dos íons sulfato por adenosina trifosfato (ATP), tanto para o processo assimilativo quanto para o processo desassimilativo (Figura 1). A enzima ATP sulfurilase catalisa a ligação do íon sulfato ao fosfato da molécula de ATP, formando adenosina fosfosulfato (AFS) com a liberação de pirofosfato inorgânico

(PPi). Na redução desassimilativa ou dissimilativa, a AFS é reduzida a sulfito (SO_3^{2-}) pela ação da enzima AFS redutase, com a liberação de adenosina monofosfato (AMP). Na redução assimilativa, outra molécula de fósforo (P) é incorporada a AFS, formando fosfoadenosina fosfosulfato (FAFS) e, a partir desse momento, os sulfatos podem ser reduzidos. Em ambos os processos, o sulfito é o primeiro produto da redução, e uma vez formado é transformado em sulfeto por intermédio da enzima sulfito redutase (MADIGAN; MARTINKO; PARKER, 2004).

As bactérias redutoras de sulfato utilizam outros compostos inorgânicos alternativos como aceptor final de elétrons, tais como: sulfitos, tiossulfato, enxofre, metabissulfito, nitrato (RABUS et al., 2006; SÉRVULO, 1991), ferro (PARK; LIN; VOORDOUW, 2007), selênio (TUCKER; BARTON; THOMPSON, 1998), cromo (LOVLEY; PHILLIPS, 1994), arsênio (MACY et al., 2000), mas nem todos estes processos de redução são acoplados ao crescimento bacteriano (MUYZER; STAMS, 2008). Além disso, as BRS podem também utilizar compostos orgânicos como aceptor final de elétrons para o crescimento, entre eles: fumarato (MUYZER; STAMS, 2008), dimetilsulfóxido (JONKERS et al., 1996), sulfonatos (LIE et al., 1996), 3-clorobenzeno (DOLFING; TIEDJE, 1991).

Estas bactérias são capazes de usar uma variedade de fontes de carbono e hidrogênio molecular (H_2) para o seu crescimento e redução de sulfato (Figura 2), entre eles: álcoois primários como metanol, etanol, propanol, butanol (KRUMHOLZ; HARRIS; SUFLITA, 2002; MUYZER; STAMS, 2008) e constituintes do petróleo como alcanos, tolueno, benzeno e hidrocarbonetos poliaromáticos (LIAMLEAM; ANNACHHATRE, 2007; YOUNG; PHELPS, 2005). Os gêneros *Desulfovibrio*, *Desulfomicrobium*, *Desulfobulbos* são capazes de oxidar compostos orgânicos parcialmente a acetato, enquanto os gêneros *Desulfobacter*, *Desulfobacterium*, *Desulfococcus* oxidam substratos orgânicos completamente a CO_2 (BARTON, 1995).

As bactérias redutoras de sulfato geralmente apresentam crescimento lento quanto comparado com outras bactérias, como, por exemplo, *Escherichia coli*. O tempo de crescimento de BRS ocorre em torno de alguns dias a semanas, dependendo das condições de cultivo (temperatura, pH, potencial de oxi-redução, fonte de carbono, aceptor de elétron) e da espécie. As BRS mesófilas crescem em temperatura ambiente, entre 28 a 30ºC, e as termófilas crescem mais rapidamente a 55ºC (POSTGATE, 1984).

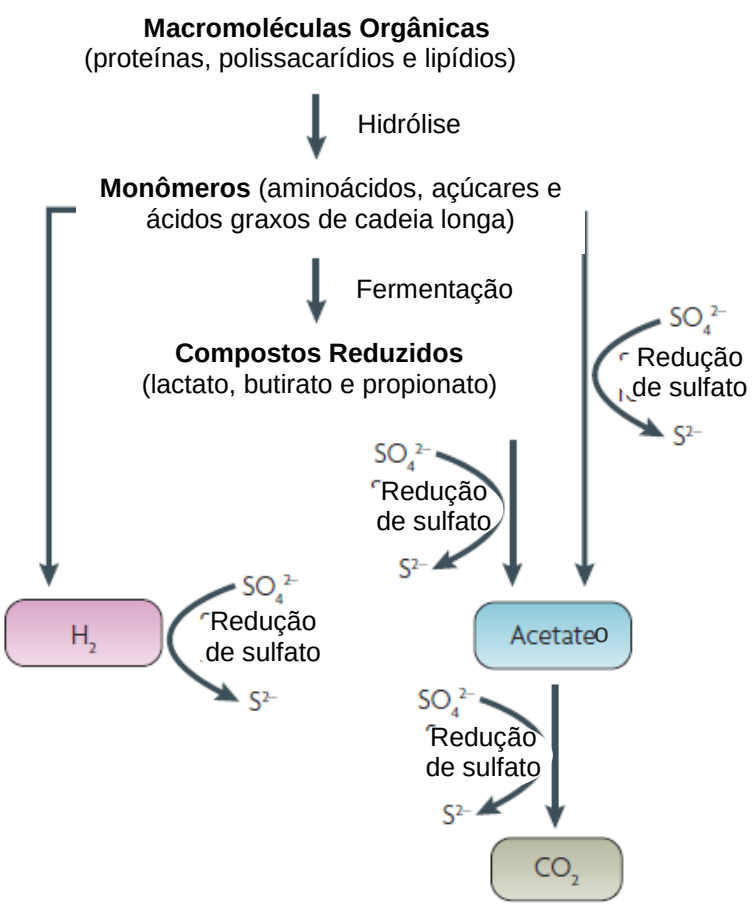

Figura 2. Degradação microbiana de compostos orgânicos em ambientes anóxicos na presença de sulfato (MUYZER; STAMS, 2008, modificado).

O pH ótimo para o crescimento de BRS isoladas ou em cultura enriquecida é em torno de 7, apresentando efeito inibidor quando menor do que 6 e maior do que 9 (FAUQUE; OLLIVIER, 2004), sendo o valor do pH fator fundamental para redução de sulfato (MIZUNO et al. 1998). Gyure et al. (1990) demonstraram o crescimento de culturas mistas contendo BRS, mas não destas isoladamente. Nesse sentindo, estes autores sugeriram que muitas BRS presentes em ambientes ácidos vivem em microambientes com pH neutro, como sedimentos ou raízes ou partículas em suspensão. Entretanto, foi relatado o crescimento de cultura mista de BRS em pH entre 3,8 e 4,2, usando glicerol como substrato (KIMURA; HALLBERG; JOHNSON, 2006), enquanto Karnachuk et al. (2009) isolaram uma linhagem do gênero *Desulfosporosinus* sp. a partir de sedimentos de uma mina de ouro com pH extremamente baixo (2,4 – 2,8) e alta concentração de metais dissolvidos. Alazard et al. (2010) isolaram e caracterizaram a espécie *D. acidiphilus* a partir de sedimentos da drenagem ácida de minas com o pH próximo de 3. Estes autores sugeriram que o uso de substratos orgânicos não ácidos, como glicerol, em culturas enriquecidas foi fundamental para obtenção de bactérias redutoras de sulfato acidófilas.

Classificação de BRS

As bactérias redutoras de sulfato foram descobertas por Beijerinck em 1895, que descreveu a espécie *Spirillum desulfuricans*. As BRS foram classificadas inicialmente em dois grandes grupos: formadoras de esporos e não formadoras de esporos. Porém, com o advento das técnicas de bioquímica e biologia molecular esses critérios mostraram suas limitações (POSTGATE; CAMPBELL, 1966; RABUS et al., 2006). Novos estudos e pesquisas foram desenvolvidos sobre as BRS tendo como base um conjunto de características como a nutrição, morfologia (vibriões, bastonetes, cocos), motilidade, temperatura, conteúdo de GC (guanina e citosina) (BARTON, 1995) e análise do RNA ribossomal 16S, permitindo que estas fossem classificadas em quatro grupos: mesófilas Gram-negativas, eubactérias termófilas Gram-negativas, Gram-positivas formadoras de esporos e arqueobactérias termófilas Gram-negativas (POSTGATE, 1984; BARTON, 1995, CASTRO; WILLIAMS; OGRAM, 2000). As BRS mesófilas Gram-negativas são as mais difundidas na natureza (BARTON, 1995), sendo o gênero *Desulfovibrio* o mais conhecido (BARTON, 1995; RABUS; HANSEN; WIDDEL, 2006).

Muyzer e Stams (2008) agruparam as bactérias redutoras de sulfato até então conhecidas em sete linhagens filogenéticas, baseada na comparação de sequências de 16S rRNA presentes em banco de dados ARB-SILVA (Figura 3). O domínio Bacteria possui cinco representantes: Deltaproteobacteria, Nitrospirae, Clostridia, Thermodesulfobiaceae e Thermodesulfobacteria, e o domínio Archaea duas: Euryarchaeota e Crenarchaeota.

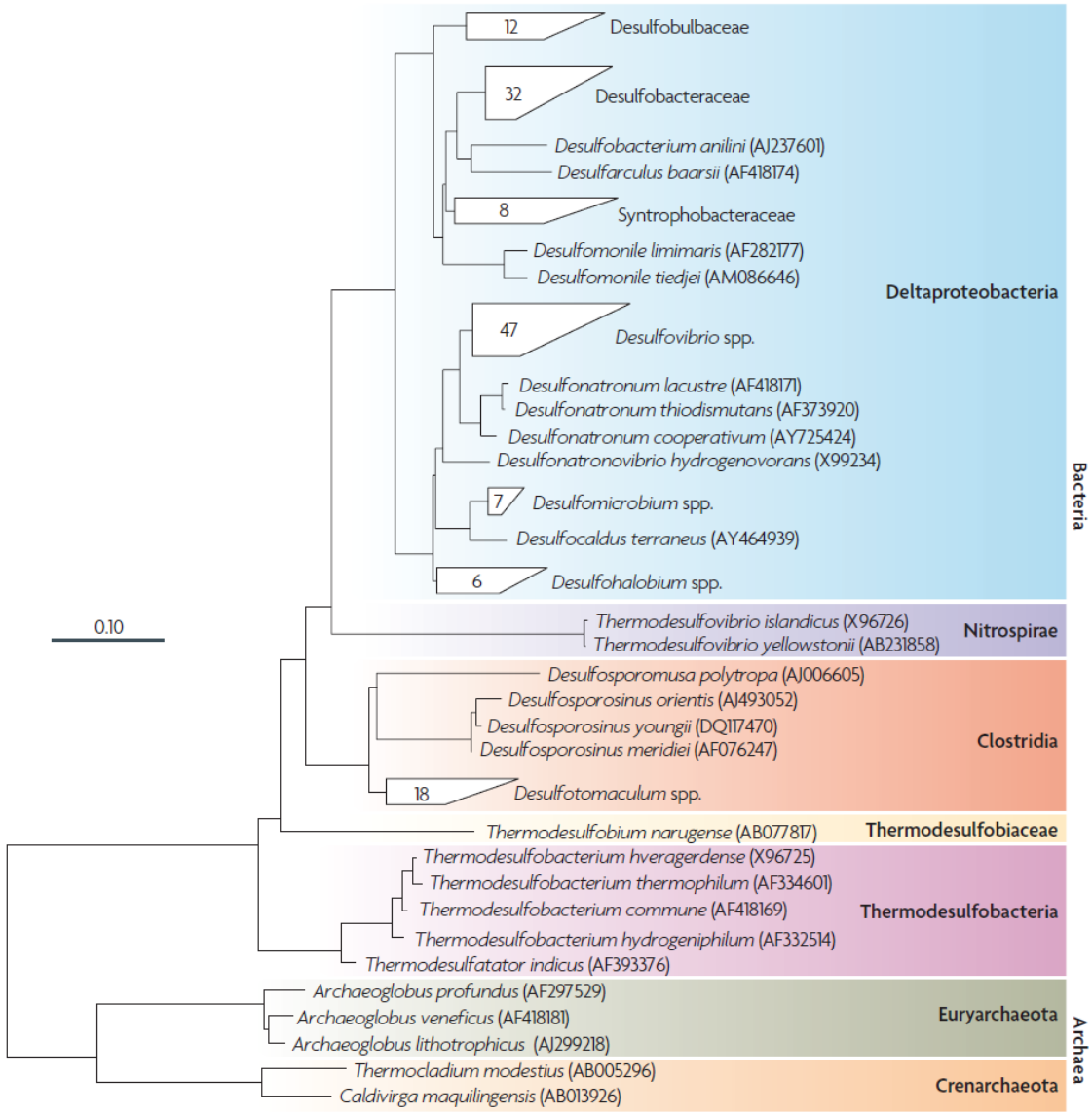

Figura 3. Árvore filogenética baseada em sequência de DNA ribossomal descrita para espécies de bactérias redutoras de sulfato. Os números dentro dos quadros indicam o número de diferentes em um grupo particular e os números em parênteses são os números de acesso ao banco de dados GenBank. A barra indica 10% de diferença entre as sequências (MUYZER; STAMS, 2008).

Morfologia de BRS

As células de bactérias redutoras de sulfato apresentam morfologia bastante diversificada, podendo ser esféricas, ovais, bastonetes ou vibrióides, com diâmetro variando de 0,4 a 3,0 µm, ocorrendo isoladas, aos pares ou, algumas vezes, formando agregados. Algumas espécies apresentam mobilidade e outras são capazes de esporular (HOLT, 1994). Os membros do gênero *Desulfovibrio* são geralmente curvos e sigmóides, mas existem exceções como, por exemplo, a espécie *Desulfovibrio desulfuricans* que têm a forma de bastonete ou víbrio. A maioria das espécies *Desulfovibrio* são monotríquia e algumas

apresentam duplo flagelo, mas já foram observadas cepas de *Desulfovibrio desulfuricans* sem flagelos. Alguns representantes deste gênero possuem predisposição a pleomorfismo, formas variáveis em culturas velhas, ou adquirem a forma helicoidal em ambientes inadequados ao crescimento (POSTGATE, 1984).

Distribuição e Ecologia de BRS

Bactérias redutoras de sulfato estão amplamente distribuídas em ambientes anóxicos, onde estejam presentes compostos orgânicos e sulfato. Assim, vários estudos têm mostrado que este grupo de micro-organismos pode ser identificado em diversos ambientes, tais como solos, sedimentos (marinho, estuário e lagos de água doce e salgada), efluentes domésticos e indústrias, e drenagem ácida de minas (CHANG et al., 2001; DEVEREUX et al., 1996; ISHII et al., 2004; PEDUZZI; TONOLLA; HAHN, 2003), rizosfera de plantas, (DAR; KUENEN; MUYZER, 2005), trato gastrintestinal de humanos e animais (DETHLEFSEN et al., 2006), fontes hidrotermais, gasodutos, e em outros ambientes onde existe a presença de oxigênio (WIERINGA et al., 2000). Além destes, as BRS também podem ser encontradas em poços de petróleo.

As bactérias redutoras de sulfato são as únicas que tem a capacidade de usar sulfato como aceptor final de elétrons. Este processo é fundamental para recirculação de enxofre e a manutenção da vida na Terra, que normalmente apresenta a forma oxidada, como sulfato e óxidos de enxofre no solo, rochas, rios e mares. Nesse sentido, é necessário que ocorra a redução biogênica das diferentes formas de enxofre para que estes sejam incorporados nas biomoléculas. A redução de sulfato inorgânico (SO_4^{2-}) a sulfeto (H_2S, HS^- e S^{-2}), inorgânico ou orgânico, e a subsequente oxidação de sulfeto a sulfato, é chamado de ciclo do enxofre (Figura 4) (BARTON, 1995).

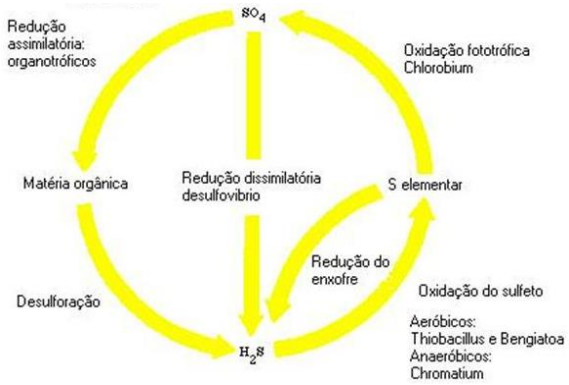

Figura 4. Ciclo biogeoquímico do enxofre (KLIMA NATURALI, 2013).

Indústria de Petróleo

Recuperação de Petróleo

Os reservatórios apresentam mecanismo primário de produção de petróleo e gás devido à pressão natural do reservatório, que fornece cerca de 5 a 10% do total da reserva de petróleo, chamada de recuperação primária, que se torna pouco eficiente após a exaustão da sua energia natural, e que, por consequência, retém grandes quantidades de hidrocarbonetos. Esse fato indica a necessidade do uso de métodos de recuperação secundária de petróleo, que envolve a estimulação de poços de petróleo pela injeção de gás natural e do uso de fluidos, para aumentar a pressão interna do reservatório e deslocar o fluxo de óleo e gás existente no poço de injeção em direção aos poços produtores (Figura 5), com a eficiência de recuperação de óleo variando de 10 a 40% (BELYAEV et al., 2004; van HAMME; SINGH; WARD, 2003).

Vários métodos de injeção de fluídos são utilizados nos reservatórios para aumentar a extração de petróleo, sendo a injeção de água do mar o principal método aplicado em campos de petróleo marítimos (*offshore).* A escolha da água do mar, em campos *offshore*, se deve aos baixos custos operacionais, tendo em vista a sua grande disponibilidade. No entanto, esta escolha pode afetar a qualidade do petróleo produzido, além de conferir um sério risco às atividades operacionais da plataforma, devido às altas concentrações de sulfato (3 g/L) presentes na água do mar (FARQUHAR, 1997; BELYAEV et al., 2004; van HAMME; SINGH; WARD, 2003). Os processos de recuperação primária e secundária possuem uma faixa de extração de óleo máxima de 40-45%; assim, estas operações de recuperação de petróleo convencional muitas vezes deixam aproximadamente dois terços de óleo no reservatório (BELYAEV et al., 2004; LAZAR; PETRISOR; YEN, 2007).

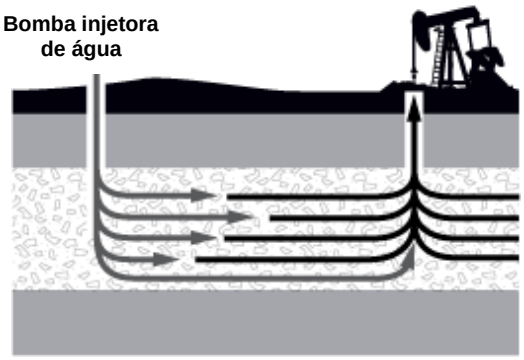

Figura 5. Recuperação secundária de petróleo através da injeção de água (adaptado de TITAN OIL RECOVERY, 2011).

Para atender a crescente demanda mundial de energia, têm sido desenvolvidos vários métodos de recuperação terciária ou avançada para remoção de óleo remanescente, tais como: injeção de surfactantes (AL-GHAMDI; NASR-El-DIN, 1996); injeção de polímeros (KERN; DASSONVILLE, 1992); tecnologia de recuperação avançada de petróleo com micro-organismos (MEOR), que utiliza micro-organismos e/ou seus produtos metabólicos na recuperação de petróleo residual (BANAT, 1995), cujos resultados ainda não chegam a ser os desejados.

Acidificação Biogênica (souring)

A utilização de água do mar como método de recuperação secundária de petróleo traz diversas vantagens econômicas e ambientais, principalmente para poços próximos a fonte, pois a água do mar representa uma fonte inesgotável. No entanto, o uso desta origina elevada produção de H_2S nos reservatórios petrolíferos, pois o sulfato, geralmente presente em altas concentrações, é metabolizado pelas bactérias redutoras de sulfato gerando o gás sulfídrico. Além disso, a injeção de água do mar nos reservatórios também promove simultaneamente a redução da salinidade e da temperatura da água de formação, estabelecendo condições de potencial redox (abaixo de -100 mV) e pH (6,0- 9,0) adequadas à atividade de BRS (LIAMLEAM; ANNACHHATRE, 2007; POSTGATE, 1984).

O sulfeto gerado pela atividade microbiana durante a produção do petróleo constitui um importante problema para as indústrias petrolíferas, devido ao desenvolvimento da acidificação biogênica, também denominada de *souring*. A acidificação biogênica implica na redução da qualidade dos hidrocarbonetos produzidos e, consequente, aumento dos custos, pela necessidade do uso de produtos químicos para a redução de H_2S até níveis aceitáveis; a redução da produtividade e da qualidade dos hidrocarbonetos produzidos, é devida à formação de compostos precipitados, como FeS (*biofouling*), que bloqueiam a produção de petróleo (plugueamento) de poços injetores e produtores; ao aumento das taxas de corrosão pelo mecanismo de despolarização catódica; e à problemas de segurança, saúde e meio ambiente, devido à toxicidade do sulfeto (HUBERT et al., 2005; KANE; SURINACH, 1997; TANG; BASKARAN; NEMATI, 2009).

Água de Produção (AP)

O método de injeção de água durante o processo de produção de petróleo gera a formação de efluente, normalmente conhecida como água de produção, sendo este o rejeito de maior volume em todo o processo de exploração e produção de petróleo. Durante a vida econômica de um poço de petróleo, o volume de água de produção pode chegar a exceder dez vezes o volume de produção de óleo (HENDERSON et al., 1999; STEPHENSON, 1992). Um campo de petróleo novo produz pouca água, em torno de 5 a 15%, entretanto, à medida que a vida econômica dos poços vai se esgotando, o volume de água pode aumentar significativamente para uma faixa de 75 a 90% (ALI et al., 1998; THOMAS, 2004).

Água de produção é um efluente complexo, de salinidade elevada, que varia de 250 mg/L a 300 g/L, com valores de dureza na faixa de 20 mg/L a 7 g/L e pH entre 4,5 a 9. A composição da AP pode variar amplamente, dependendo do tipo de campo e da sua idade, origem e qualidade do óleo, bem como do procedimento usado para sua extração (SCHLUTER, 2007; SOUZA, 2007). Os compostos que, normalmente, compõem esta água são: óleo disperso e dissolvido; sólidos dissolvidos totais, sendo que a concentração de cloreto de sódio pode corresponder até 80% deste total; sais minerais dissolvidos (Na^{+}, K^{+}, Fe^{2+}, Cl^{-}); metais pesados (Cd, Pb, Hg, Zn); traços de metais radioativos (K^{40}, U^{238}, Ra^{226}), compostos orgânicos naturais (alifáticos, aromáticos, polares e ácidos graxos), graxas e asfaltenos; produtos químicos adicionados para prevenir e/ou tratar problemas operacionais, tais como biocidas, anti-incrustantes, anti-espumantes e inibidores de corrosão; e gases dissolvidos, incluindo CO_2 e H_2S (ALMEIDA, 2004; HANSEN; DAVIES, 1994; OLIVEIRA; OLIVEIRA, 2000; TELLEZ et al., 2002).

A reutilização da água produzida é justificada como alternativa de manejo, uma vez que sua disposição sem tratamento prévio acarreta sérios danos ambientais (VOORDOUW; JACK, 2005). Entretanto, estudos realizados em campos do Mar do Norte demonstraram que o processo de recuperação secundária do petróleo, através da injeção da água do mar e da reinjeção da água produzida potencializa o risco de intensificação da acidificação biogênica (*souring*), uma vez que convergem para elevados valores nas concentrações de sulfato, ácidos graxos voláteis, salinidade e de bactérias redutoras de sulfato para a atividade microbiana, além de reintroduzir micro-organismos já adaptados às condições do reservatório (LYSNES et al., 2009).

Água produzida representa uma das principais amostras oriundas de campos de petróleo para análises física, química e biológica, sendo esta fundamental para caracterização de comunidade microbiana e suas relações ecológicas.

Biocorrosão

A indústria de petróleo se destaca como uma das que mais é afetada pelo fenômeno corrosivo, em virtude da grande quantidade de material metálico utilizado, muitas vezes recobrindo grandes extensões de difícil acesso. A corrosão é um fenômeno químico ou eletroquímico de superfície que resulta na deterioração dos materiais, normalmente metálicos, tanto expostos a ambientes aquosos ou ligeiramente úmidos (corrosão eletroquímica), quanto secos (corrosão química) (GENTIL, 2003). A corrosão é um processo de oxidação que leva a dissolução do metal na região anódica e, simultaneamente, um processo catódico complementar, resultando na redução de algum componente do meio, através da reação catódica (VIDELA, 2003).

O processo corrosivo pode ser influenciado pela atividade microbiana, especialmente quando os micro-organismos estão em estreito contato com a superfície do metal, formando comunidades que atuam em sinergismo ou sinergia, sendo capazes de afetar os processos eletroquímicos através do metabolismo cooperativo não visto em espécies de forma individual (DOWLING et al., 1992), sendo esta organização conhecida como biofilme (*biofouling*). O biofilme é uma estrutura complexa, constituída de agregados celulares inseridos numa matriz de exopolissacarídeo (EPS) e canais intersticiais, por onde ocorre a passagem do fluido circulante (LEWANDOWSKI et al., 1995). A presença física do biofilme na superfície do metal e/ou sua atividade metabólica conduz a importantes modificações da interface metal/solução, formando uma barreira entre o metal e o líquido circundante (VIDELA, 2003). A deterioração do metal resultante é conhecida como biocorrosão ou corrosão microbiologicamente induzida (CMI).

Os micro-organismos podem participar ativamente nesse processo de corrosão, causando a dissolução do metal. Porém, a CMI não é um tipo diferente de ação do processo de corrosão, mas sim um adjuvante, pois na maioria dos casos promove a aceleração da cinética das reações, sem alterar a natureza eletroquímica do processo corrosivo (COETSER; CLOETE, 2005; ORNEK et al., 2002). Os micro-organismos participam do processo de corrosão de forma ativa, produzindo substâncias corrosivas, originadas durante seu crescimento ou metabolismo, as quais podem ser de natureza química diversa, como ácidos,

álcalis, sulfeto entre outros, transformando um meio originalmente inerte em agressivo (VIDELA, 2003). Dentre os vários micro-organismos reconhecidamente envolvidos em casos de biocorrosão, as BRS são consideradas as principais. Diversos modelos têm sido propostos para explicar os mecanismos pelos quais as BRS podem influenciar no processo corrosivo, onde a atividade de redução do sulfato está de alguma forma relacionada (Tabela 1).

A corrosão provocada pelo sulfeto produzido pela atividade das BRS é mais agressiva do que pelo sulfeto de origem química, demonstrando a influência da presença de BRS e sua atividade metabólica no processo corrosivo (MCNEIL; LITTLE, 1990; THOMAS; EDYVEAN; BROOK, 1988). O estudo realizado por Videla et al. (1999), utilizando métodos de análise fisico-química, entre eles difração de raios-X, análise de microssonda eletrônica e microscopia eletrônica de varredura, demonstrou que os filmes de sulfeto formados sobre o aço na presença de BRS (sulfeto biótico), *Desulfovibrio alaskensis*, foram mais aderentes do que aqueles formados em meio estéril contendo sulfeto comercial (sulfeto abiótico).

Tabela 1. Os mecanismos sugeridos de corrosão de metal pelas BRS.

Processo corrosivo/substância	Referência
Despolarização catódica pela hidrogenase	BRYANT et al., 1991.
Despolarização anódica	CROLET, 1992.
Sulfeto	LITTLE; WAGNER; LEWANDOWSKI, 1998.
Sulfeto de ferro	KING; WAKERLEY, 1973.
Um composto de fósforo volátil	IVERSON; OHLSON, 1983.
Exopolímeros ligados ao ferro	BEECH; GAYLARDE, 1999.
Corrosão induzida por tensão sulfeto	EDYVEAN et al., 1998.
Hidrogênio induzindo a formação de rachaduras ou bolhas	EDYVEAN et al., 1998.

O mecanismo clássico de corrosão anaeróbica foi proposto por Von Wolzogen Kuhr e van der Vlugt (1934), tendo como base a desporalização catódica, que é alcançada pela oxidação metabólica de H_2 de bactérias redutoras de sulfato (Figure 6), de acordo as seguintes reações:

$$4Fe \leftrightarrow 4Fe^{2+} + 8e^{-} \text{ (reação anódica)} \quad (1)$$

$$8H_20 \leftrightarrow 8H^{+} + 8OH^{-} \text{ (dissociação da água)} \quad (2)$$

$$8e^{-} + 8H^{+} \leftrightarrow 8H \text{ (reação catódica)} \quad (3)$$

$$SO_4^{2-} + 8H \leftrightarrow S^{2-} + 4H_20 \text{ (desporalização catódica)} \quad (4)$$

$$Fe^{2+} + S^{2-} \leftrightarrow FeS \text{ (produtos de corrosão)} \quad (5)$$

A atividade bacteriana e, principalmente das BRS, é responsável por mais de 75% da corrosão em poços de petróleo em produção e por mais de 50% das falhas em dutos e cabos (WALCH, 1992). Estruturas metálicas, incluindo plataformas, sistemas de dutos, fundos dos tanques, bombas e peças de outros equipamentos podem apresentar falhas em áreas onde a corrosão de natureza microbiológica ocorra. Se uma falha ocorrer em dutos ou no fundo dos tanques de estocagem, navios e plataformas, o fluido que venha a ser liberado nestes ambientes pode causar sérias consequências ambientais, em decorrência da contaminação do solo e/ou água em grandes proporções. Da mesma forma, se uma falha ocorrer em uma linha de gás ou de água de alta pressão, a conseqüência para funcionários operadores pode ser de injúria ou até morte, tendo em vista que o H_2S é um gás incolor e altamente tóxico, apresentando alta letalidade para o homem, mesmo em baixas concentrações. Qualquer um desses possíveis acidentes exemplificados anteriormente envolve custos elevados para reparação ou substituição de peças, com fator adicional de procedimentos para a remoção do H_2S biogênico dos óleos e gases acidificados, aumentando os custos desses produtos, além de multas de ordem ambiental e processos trabalhistas (HUBERT et al., 2005; JAVAHERDASHTI, 1999).

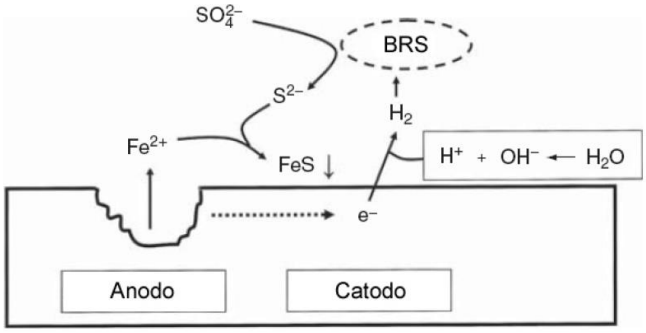

Figura 6. Modelo de corrosão realizada pelas bactérias redutoras de sulfato (BRS) (BARTON; FAUQUE, 2009).

Existem algumas formas de controlar a produção de gás sulfídrico em poços de petróleo, sendo os antimicrobianos (biocidas) ou inibidores metabólicos os mais usados, com a finalidade de destruir ou inibir os micro-organismos produtores de gás sulfídrico, respectivamente. Esse procedimento ocorre adicionando-se biocidas (glutaraldeído e cocodiamina) juntamente com a água de injeção (CARVALHO, 2010). Os biocidas, por

serem usados em altas concentrações, apresentam custos elevados e, na maioria das vezes, não são capazes de resolver o problema devido ao surgimento de micro-organismos sulfetogênicos resistentes. Nemati (2001) relatou a presença de bactérias redutoras de sulfato resistentes à concentração de 500 mg/L de biocidas a base de cocodiamina. A cultura mista de BRS (APB55) apresentou resistência ao biocida formado por glutaraldeído e compostos de amônia quaternária, que é injetado diariamente nos poços de petróleo em estudo, que na maioria das vezes não são capazes de resolver o problema devido ao surgimento de micro-organismos sulfetogênicos resistentes, dificultando ou impossibilitando o seu controle. Além disso, os biocidas são utilizados de forma empírica, muitas vezes destruindo os micro-organismos que exercem efeitos benéficos à produção de petróleo (SANTOS; QUINTELLA; ALMEIDA, 2009).

O último estudo desenvolvido pela Corrosion Costs and Preventive Strategies (2010) para avaliar o impacto da corrosão na economia nos Estados Unidos foi realizado de 1999 a 2001, sendo conduzido pela empresa CC Technologies Laboratories, em parceria com Federal Higway Administration (FHWA), conhecida como a Sociedade da Corrosão, e a Nace International (NACE). Esse estudo indicou um gasto anual de aproximadamente US$ 276 bilhões, representando 3,1% do Produto Interno Bruto (PIB), sendo as atividades de produção e exploração de óleo e gás responsáveis por 8% (US$1.4 bilhões) e as de refino de petróleo por 21% (US$3.7 bilhões). No Brasil, segundo avaliação realizada pela Associação Brasileira de Corrosão (2010), os custos relacionados à corrosão são de aproximadamente US$ 15 bilhões por ano, representando 3% do produto interno bruto. Segundo Flemming (1996), a corrosão microbiologicamente induzida ou biocorrosão é responsável por aproximadamente 20% do total do processo corrosivo.

Biorremediação de metais pesados

O processo de redução biológica de sulfato tem sido proposto como uma alternativa econômica e ambiental para biorremediação de áreas impactadas por metais pesados (GIBERT et al., 2004; ICGEN; HARRISON, 2006), sendo a drenagem ácida de minas (DAM) a mais referenciada, por ser um dos mais graves impactos ambientais associados à atividade de mineração.

A DAM é a solução aquosa ácida gerada quando minerais presentes em resíduos de mineração são oxidados espontaneamente em presença de oxigênio (O_2) e água (H_2O), produzindo ácido sulfúrico que dissolve metais presentes no solo e rocha (LUPTAKOVA;

KUSNIEROVA, 2005; MUYZER; STAMS, 2008; BANKS et al., 1997). O sulfato (SO_4^{2-}) representa a família de minerais que mais sofre esse processo de redução, sendo a pirita (dissulfeto de ferro, FeS_2) o mais abundante de todos os minerais sulfetados. A oxidação da pirita pode ser descrita com as reações a seguir (BANKS et al., 1997).

$$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2Fe^{2+} + 4SO_4^{2-} + 4H^+ \quad (1)$$

A oxidação de ferroso (ferro II ou Fe^{2+}) a férrico (ferro III ou Fe^{3+}) consome os prótons produzidos na reação 1, de acordo a reação 2 (BANKS et al., 1997):

$$4Fe^{2+} + 4H^+ + O_2 \rightarrow 4Fe^{3+} + 2H_2O \quad (2)$$

Ferro III pode atuar como um aceptor de elétrons para oxidação de mais pirita ou sofrer hidrolise. Em ambos os processos ocorre a liberação de prótons, de acordo as reações 3 e 4 (BANKS et al., 1997):

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15\ Fe^{2+} + 2SO_4^{2-} + 16H^+ \quad (3)$$

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+ \quad (4)$$

O resultado das reações é a produção de ácido (reação 5) (BANKS et al., 1997). Esta condição ácida resulta na dissolução de mais metais a partir da mineração (van HOUTEN; LETTINGA, 1995).

$$4FeS_2 + 14H_2O + 15O_2 \rightarrow 4Fe(OH)_3 + 8SO_4^{2-} + 16H^+ \quad (5)$$

A combinação da remoção da acidez, dos metais e do sulfato torna o processo de redução biológica o mais promissor para o tratamento de drenagem ácida de minas e para recuperação de metais. Este processo é baseado na produção biológica de sulfeto e alcalinidade da solução pelas bactérias redutoras de sulfato. As BRS, sob condições anaeróbicas, utilizam compostos orgânicos e H_2 como fonte de energia e sulfato como aceptor final de elétrons, gerando sulfeto e íons bicarbonato ou hidróxido, de acordo as equações 6 e 7 (KAKSONEN; PUHAKKA, 2007; KIM; KILBANE; CHA, 1999).

$$C_2H_4O_2 + S0_4^{2-} \rightarrow 2HCO_3^- + H_2S \quad (6)$$

$$8H_2 + 2SO_4^{2-} \rightarrow H_2S + HS^- + 5H_2O + 3OH^- \quad (7)$$

O sulfeto de hidrogênio reage com metais, como Zn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, Hg^{2+}, Pb^{2+}, Cd^{2+}, segundo a reação 8 (M^{2+} significa o metal), para formar precipitados insolúveis, que podem ser facilmente separados da solução (BENEDETTO et al., 2005; KAKSONEN; PUHAKKA, 2007).

$$H_2S + M^{2+} \rightarrow MS + 2H^+ \quad (8)$$

Esta reação de precipitação libera prótons que aumenta a acidez da solução. Porém, os íons bicarbonato e hidróxido produzidos durante a oxidação dos doadores de eléltrons (reações 6 e 7) neutralizam a acidez da solução (reações 9 e 10), para valores de pH na faixa de 6 a 7 (DVORAK et al, 1992; KAKSONEN; PUHAKKA, 2007). A capacidade de neutralizar a solução também resultará na precipitação de alguns íons metálicos como hidróxidos insolúveis (DVORAK et al, 1992).

$$HCO_3^- + H^+ \rightarrow CO_2 + H_2O \quad (9)$$

$$OH^- + H^+ \rightarrow H_2O \quad (10)$$

Estudo realizado por Maree e Strydom (1987) demonstrou a remoção biológica de sulfato de efluentes industriais em reator, onde as BRS foram capazes de retirar 89% de sulfato e reduzir a concentração de mais do que 50% dos metais (Ca, Cr, Cd, Mn, Ni, Zn, Fe), com o pH mudando de 5,0 (inicial) para 8,1 (final). Dvorak et al. (1992) também reportaram, em uma pesquisa piloto em reator, que as bactérias redutoras de sulfato reduziram mais de 95% da concentração dos metais (Cd, Fe, Mn, Ni, Zn) e neutralizaram completamente a acidez do efluente. O sistema de tratamento de efluentes em biorreator contendo um biofilme de BRS mostrou ser eficiente na remoção e precipitação de metais mesmo em baixo valor de pH (1,7). Além disso, o sistema tolerou altas concentrações de metais pesados (>270 mg/L de Cd), com capacidade de remoção variando entre 95 a 99% (XIE, 1993). Experimento em coluna de fluxo contínuo contendo BRS para o tratamento de efluentes apresentou uma capacidade de extrair mais de 99% de cádmio, cobre e zinco, e de 87% de níquel, neutralizando o valor do pH do efluente de 3 para 7 (KIM; KILBANE; CHA, 1999). Estudo piloto realizado por Jong e Parry (2003) demonstrou que as BRS foram eficazes no tratamento de água ácida em um biorreator de coluna anaeróbia, com habilidade de remover mais de 80% de sulfato e acima de 97,5% de cobre, zinco e níquel, e mais de 82% de ferro, alterando o pH de, aproximadamente, 4,0 para 7,2. Esse processo apresentou menores custos quando comparado com o tratamento de neutralização por adição substância alcalina.

O tratamento convencional para remoção do sulfato e correção da acidez é a adição de chumbo, bário, alumínio e cálcio, sendo este último o mais utilizado. O hidróxido de cálcio ou cal hidratada, $Ca(OH)_2$, e óxido de cálcio ou cal virgem, CaO, reagem com o sulfato (SO_4^{2-}) presente na água ácida ou na drenagem ácida de minas formando a gipsita ($CaSO_4.2H_2O$), que é precipitada. Porém, esse tratamento não remove eficientemente os níveis de sulfato, deixando resíduos na faixa de 1,4 a 1,6 g/L, que é acima do limite máximo permitido pela legislação (0,25 g/L). Ainda esse tratamento não tem apresentado bons resultados na redução dos níveis de sulfato, pois os metais precipitados podem ser ressolubilizados devido à capacidade de solubilização do sulfato de cálcio, e finalmente, esse tratamento tem sido considerado de alto custo e o rejeito produzido é tóxico ao meio ambiente (ELLIOT; RAGUSA; CATCHESIDE, 1998; LUPTAKOVA; KUSNIEROVA, 2005).

O processo de redução biológica de sulfato possui a capacidade de reduzir os níveis de sulfato e metais, além de neutralizar o pH da solução, tornando-se uma alternativa econômica e ambiental promissora para remediação de drenagem ácida de minas e para recuperação de metais. Fontes alternativas de carbono têm sido usadas para o crescimento e redução de sulfato pelas bactérias redutoras de sulfato, no sentido de reduzir os custos de produção. Com isso, a glicerina bruta pode se tornar um substrato potencial para biorremediação de áreas impactadas por metais pesados.

Glicerina Bruta (GB)

A glicerina bruta é o principal co-produto da produção do biodiesel, sendo composta de glicerina, ácidos, ésteres, álcalis e álcoois, apresentando baixo grau de pureza, e tendo geralmente a composição de 40 a 90% de glicerina, 8 a 50% de água, menos de 2% de metanol e até 10% de sais (QUINTELLA; CASTRO, 2009). O termo glicerina aplica-se aos produtos comerciais purificados que contém glicerol com diferentes graus de pureza, normalmente, contendo pelo menos 95% de glicerol, enquanto o nome glicerol é somente aplicado ao composto químico puro 1,2,3-propanotriol (APPLEBY, 2006; MOTA; SILVA; GONÇALVES, 2009). O glicerol é um álcool que se apresenta como um líquido viscoso, inodoro, incolor e com sabor doce (MORRISON, 1994).

O glicerol apresenta uma ampla variedade de aplicação industrial devido as suas características, tais como não toxicidade e ausência de cor e odor. É atualmente um dos ingredientes mais utilizados na indústria farmacêutica na composição de cápsulas,

supositórios, anestésicos, xaropes e emolientes para cremes e pomadas, antibióticos e anti-sépticos. Na indústria alimentícia é utilizado em preparações de molhos e sobremesas, assim como umectante de alimentos (MORRINSON, 1994; NAE, 2005). O glicerol também é um importante agente crioprotetor, uma vez que não permite a formação de cristais de gelo em meio aquoso e o consequente rompimento das células, mantendo sua viabilidade durante os processos de congelamento (TSURUTA; ISHIMOTO; MASUOKA, 1998). Outras aplicações incluem o emprego como lubrificante de máquinas processadoras de alimentos, na fabricação de dinamite, no processamento de tabaco e como lubrificante na indústria têxtil (MORRISON, 1994; BRISSON et al., 2001; NAE, 2005).

A glicerina derivada da produção do biodiesel possui baixo valor agregado, devido às impurezas decorrentes da produção do biodiesel (JOHNSON; TACONI, 2007; THOMPSON; HE, 2006). Esses resíduos tornam a GB inadequada para uso direto na indústria e a sua purificação tem um custo mais elevado do que a obtenção da glicerina por outras fontes (OOI et al., 2004; QUINTELLA et al., 2006). Além disso, tem-se observado a redução do preço internacional de glicerina purificada (MFRURAL, 2009). No Brasil, o preço que em 2005 chegou a R$ 3,00 Kg, está em torno de R$ 0,60 (BIODIESELBR.COM, 2010), sendo a queda de preço atribuída ao aumento da oferta no mercado em razão da produção de biodiesel (QUINTELLA; CASTRO, 2009). Assim, acredita-se que o mercado químico atual não terá condições de absorver tal oferta e novas aplicações deverão ser desenvolvidas (QUINTELLA et al., 2006).

Novas pesquisas estão sendo propostas tendo como base a transformação química e a conversão microbiana por processos biotecnológicos, visando converter a GB em produtos de maior valor econômico (PYLE; GARCIA; WEN, 2008; YAZDANI; GONZALEZ, 2007), como, por exemplo: (1) a conversão de glicerol em propileno glicol e acetona por meio de processos termo-químicos (CHIU et al., 2006; DASARI et al., 2005); (2) a eterificação de glicerol com álcoois (metanol ou etanol) ou alcenos (isobuteno) produz compostos de menor polaridade e viscosidade e, por conseguinte, de maior volatilidade, que podem ter apropriado propriedades como aditivos para combustíveis e solventes (KARINEN; KRAUSE, 2006); (3) o uso de de GB como flúido para recuperação secundária de petróleo em célula micro-reservatório. Os resultados demonstraram que GB remove parafinas lineares e ramificadas com fator de recuperação de 80%, dobrando o fator de recuperação, quando comparado com polímero (óxido de polietileno) e detergente (lauril sulfato de sódio), sendo o sucesso do método atribuído à ação surfactante dos triglicerídeos e ácidos graxos encontrados na glicerina bruta (QUINTELLA et al., 2006); (4) a conversão microbiana (fermentação) de

glicerol em 1,3-propanodiol, que pode ser utilizado como componente básico para a produção de poliésteres (BARBIRATO et al., 1998; ITO et at., 2005); (5) a conversão microbiana de glicerol a biossurfactante. A utilização de 5 g/L de GB, contendo aproximadamente 1 g/L de glicerol, pela linhagem *Pseudomonas* sp. Slim03 produziu cerca de 0.3 g/L de ramnose. Este biossurfactante foi capaz de reduzir as tensões superficial da água de 72.29 mN/m para 30.81 e interfacial de óleo cru de 17,16 mN/m para 1,88 mN/m; de formar e estabilizar emulsões em óleos com diferentes viscosidades; e de manter sua atividade frente a fatores ambientais, tais como temperatura (4-120ºC), pH (2-12) e salinidade (5-25%). Estas características apresentam vantagens importantes quando comparado como os surfactantes sintéticos, tais como baixa toxicidade, biodegradabilidade e atividade específica em condições extremas de temperatura, pH e salinidade. A conversão biológica de GB pode gerar valor agregado ao resíduo de síntese, ramnose, que possui potencial aplicação para as industriais de petróleo, higiene e limpeza, alimentos, e de cosméticos (SANTOS; QUINTELLA; ALMEIDA, 2009); (6) outros produtos oriundos da fermentação microbiana como butanol (BIEBL, 2001), ácido propiônico (BORIES et al., 2004), etanol e formato (JARVIS et al., 1997), ácido succínico (LEE et al., 2001), hidrogênio e etanol (ITO et al., 2005) foram também obtidos utilizando GB como fonte de carbono.

O glicerol é abundante nos ambientes naturais, uma vez que ele é o componente estrutural de membrana de diversos organismos (WANG et al., 2001). Devido à sua ampla ocorrência na natureza, muitos micro-organismos conhecidos podem utilizar glicerol como única fonte de carbono e energia. Dessa forma, processos de produção biotecnológica, baseados na convenção da GB, demonstram que esta substância química é uma fonte de carbono nova, abundante e promissora para microbiologia industrial.

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CAPÍTULO 2: CRUDE GLYCEROL AS A SUBSTRATE FOR SULFATE-REDUCING BACTERIA FROM A MATURE OIL FIELD OF BAHIA, BRAZIL AND ITS IMPACT ON SULFIDOGENESIS

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Abstract

Crude glycerol (CG) is a waste product formed during biodiesel production. Several attempts have been made to increase the economic value of this carbon source by using it for industrial processes, like enhanced oil recovery. However, CG may also be an attractive electron donor for microbial redox processes such as sulfate reduction. The aim of this study was to evaluate the sulfide production of sulfate-reducing bacteria (SRB) indigenous to oil reservoirs using CG as substrate. Samples of CG were obtained from biodiesel production, processing castor beans, soybeans, cotton and oils and waste fats. Tests were performed in Postgate medium, with different types and concentrations of CG, and a mixed inoculum of SRB from produced water of a mature oil well of Bahia (Brazil). The experiment was monitored by measuring the concentration of sulfide using a colorimetric method. Results show that the SRB grew and produced more than 250 ppm sulfide (depleting all available sulfate) at CG concentrations below 3%. However, at concentrations of CG above 3% the biogenic production of sulfide was reduced to approximately 30 ppm. The study shows that crude glycerol will likely stimulate SRB in oil fields, whenever CG is present at lower concentrations. Inhibitive concentrations of CG would probably not be maintainable throughout an oil reservoir at all times. On the other hand our results suggest CG as a cost-effective electron donor and carbon source for microbial reduction processes in the field of environmental biotechnology, like the precipitation of metals based on biologically formed sulfide.

Keywords Souring Sulfate-reducing bacteria Crude glycerol Inhibition

Introduction

Crude glycerol (CG) is the main by-product of biodiesel production. It accounts for up to 10% of the product and contains between 40 and 90% pure glycerol (Hansen et al. 2009; Hazimah et al. 2003). The growth of biodiesel production has resulted in an excess of glycerol and consequently in a decline of the market price for glycerol (Sandun et al. 2007; Yazdani and Gonzalez 2007). This glycerol is considered as a waste due to the presence of impurities such as alcohol, salts and heavy metals, that makes this by-product unsuitable for direct industrial use and its purification has a higher cost than obtaining it from other sources (Johnson and Taconi 2007; Ooi et al. 2004). This scenario indicates that the commercial viability of biodiesel calls for new and sustainable solutions for the consumption of this extra amount of crude glycerol (López et al. 2009; Pyle et al. 2008; Silva et al. 2009).

New strategies are being developed with the aim to increase the economic value of this by-product (López et al. 2009; Silva et al. 2009; Yazdani and Gonzalez 2007), such as the chemical conversion of glycerol to propylene glycol (Chiu et al. 2006); the bioconversion (fermentation) of glycerol to compounds of industrial interest, like 1,3-propanediol or ethanol (Ito et al. 2005; van Gelder et al. 2011); and the use of CG as fluid for oil recovery in the micro-reservoir cell. Latter could achieve an oil recovery factor of 80% and can thus be regarded as a promising method for the petroleum industry (Quintella et al. 2006). However, there is still limited knowledge about the microorganisms utilizing the injected CG in the oil well and what products are formed during the process. The injection of crude glycerol in oil wells could possibly stimulate the growth and sulfide production of SRB, that are widely distributed in anoxic environments, like oil reservoirs (Muyzer and Stams 2008; Postgate 1984). Microbial sulfide production in oil reservoirs has been a major problem in the petroleum industry (called souring), lowering the productivity and quality of produced hydrocarbons, increasing corrosion and causing safety, health and environmental problems (Hubert et al. 2005; Coetser and Cloete 2005). A potentially stimulating effect of CG on SRB that are indigenous to oil reservoirs would therefore intensify the problem of souring and thus have a negative impact on its usability for enhanced oil recovery purposes.

The aim of this study was to evaluate the use of crude glycerol by oil reservoir inhabiting sulfate-reducing bacteria. A mixed culture of sulfate-reducing bacteria isolated from a mature oil field (Bahia, Brazil) was incubated with different sorts of crude glycerol from biodiesel production as only carbon and energy source and the biological formation of sulfide was followed over time.

Materials and Methods

Produced Water (PW)

The samples of produced water were collected at a mature oil field of Bahia (Brazil) in 2009. The reservoir temperature is proximally 44°C, the initial pressure is 55 bar (Estublier et al. 2011) and the salt concentration of collected samples was 3.5%. The pH and sulfide concentration of the produced water were analyzed after centrifugation. The salt composition was determined using X-ray fluorescence (Table 1). The samples were evaporated in an oven and the solid phase was used to analyze the salts (Bernardez et al. 2013).

Table 1 Salt composition of produced water samples collected at a mature oil field of Bahia (Brazil)

Salt	Concentration (ppm)
Sr	1.51
Rb	0.58
Fe	0.12
Mn	1.09
Ca	110.60
K	5.95
Ba	3.29
Te	0.89
Cs	0.37
Sb	0.29
Sn	0.20
Th	0.12

Quantification and selection of SRB

The quantification of sulfate-reducing bacteria from produced water samples was done by the Most Probable Number (MPN) method, according to the American Society of Testing and Material (2002). MPN estimates were calculated from statistical tables (APHA, 1973). Serial dilutions were performed in modified Postgate E medium, containing the following (g/l): NaCl, 35.0; $C_6H_5Na_3O_7$, 6.38; $MgCl_2.6H_2O$, 1.83; KH_2PO_4, 0.5; NH_4Cl, 1.0; Na_2SO_4, 1.0; $CaCl_2$, 1.0; yeast extract, 1.0; ascorbic acid, 0.1; sodium thioglycolate, 0.01; $FeSO_4.7H_2O$, 0.5; agar, 1.5; sodium lactate, 1.75 ml (60%, w/w); resazurin, 2.0 ml (0.025%, w/v). The pH was

adjusted to 7.5-8.0 using HCl or NaOH and the medium was sterilized at 121°C for 20 min. The samples were incubated in an anaerobic chamber (Bactron VI, Shellab, Sheldon Manufacturing Inc.) at 38°C for 21 days. The growth of sulfate-reducing bacteria was indirectly followed by the formation of iron sulfides (appearing black), only occurring if sulfate reduction takes place.

Aliquots of 0.1 ml of positive MPN cultures were transferred to solid Postgate medium (agar, 15 g/l), and streaked using the spread plate method (Clesceri et al. 1998). The plates were incubated in the anaerobic chamber at 38°C. The selection of SRB was made based on macroscopic features and the formation of black precipitates in the colonies. A mixed culture of sulfate-reducing bacteria, called APB55, was composed by the selection of five different pure colonies. The morphological characteristics of the particular strains in the mixed culture were confirmed by microscopy.

Identification of sulfate-reducing bacteria (SRB)

Fluorescence in-situ hybridization (FISH) technique was used to identify the presence of sulfate-reducing bacteria in APB55, according to standard procedures (Daims et al. 2005). We used the indocarbocyanine (Cy3)-labeled 16S rRNA oligonucleotide probes SRB-385 (5'-CGGCGTCGCTGCGTCAGG-3') for the SRB group of the δ-Proteobacteria (Amann et al. 1992), DSV-698 (5'-GTTCCTCCAGATATCTACGG-3') for the Desulfovibrionaceae family (Manz et al. 1998) and SRB-129 (5'-CAGGCTTGAAGGCAGATT-3') for the *Desulfobacter* genus (Devereux et al. 1992). The total amount of cells was visualized by using DAPI (4',6-diamino-2-phenyl indole), a DNA-binding stain. Fluorescence microscopy equipped with two sets of filters (U-MWU2 - 330/420nm and U-MSWG2 - 480/590nm) was used to detect DAPI and Cy-3 stained cells respectively (Santos et al., 2010). The slides were analyzed using a microscope (Olympus®, BX51) coupled to a camera (Olympus®, Q-Color). Image analyses were carried out using the software Image Pro-Plus 5.1.

Crude glycerol (CG)

The samples of crude glycerol were collected in the Petrobras Biofuel plant, situated in Candeias (Bahia, Brazil). Four samples of CG were obtained from biodiesel production using vegetable oils: castor beans, soybeans and cotton, and oils and waste fats (OWF) as raw materials. The CG samples were sterilized in the oven at 170ºC for 1 hour (Gherna 1981). The chemical composition of the crude glycerol samples was determined, according to the analytical methods mentioned below.

Evaluation of sulfide production

The assay of biogenic sulfide production was carried out in test tubes containing 20 ml of modified Postgate E medium containing crude glycerol as sole carbon and energy source. Four different samples of CG from biodiesel production were used in concentrations of 1-5% (w/v). Sterilized commercial glycerol (99.5%, Merck) was used as a control. Inocula of 10^3-10^5 cells/ml of APB55 were added to the test tubes. Epifluorescent microscopy counting acridine orange (3,6-bis dimethylamino acridinium chloride) stained cells was carried out to standardize the inocula of APB55 (Francisco et al. 1973). Before staining, the culture was washed with Tween 80 (0.1%) and centrifuged at 7500 g for 10 minutes. The slides were analyzed with an Olympus® BX51 microscope. The test tubes were incubated in an anaerobic chamber at 38°C for 96 hours. Aliquots of the incubated tubes were collected to analyze the sulfide production. The tests were performed with duplicates. The sulfide production was expressed by using the arithmetic mean and the range of duplicates.

Analytical methods

For the pH measurements a pH-Fix 0-14 tape (Macherey-Nagel) was used. Sulfide was determined with the N,N-dimetil-p-fenilenediamina method (Trüper and Schlegel 1964). The sulfide content of the cultures was measured at 660 nm on a spectrophotometer (PerkinElmer, Vitor 1420). The standard curve was prepared from the dilution of a standard solution of sodium sulfide and results were expressed in parts per million (ppm). The glycerol concentration of the CG samples was determined by an enzymatic assay measuring triglycerides (Doles) that is also sensitive for glycerol and the absorbance was measured at 510 nm. Aldehydes were quantified by extracting them with acetonitrile (Merck) followed by a derivatization step with an acid solution of 2,4-dinitrophenylhydrazine. The carbonyl hydrazones were separated on a X-Terra MS C18 column (2.1 x 250 mm) and analyzed in an Agilent 1100 LC-DAD-MS system (Agilent, Waldbronn) Bruker Esquire 3000 plus (Bruker, Billerica). The mobile phase was water (25%) and acetonitrile (75%). Quantification of the aldehydes, as the respective hydrazones, was done by external calibration curves, and the absorbance of compounds was monitored at 365 nm (Bastos and Pereira 2010).

Results

Quantification and identification of SRB

The sulfide concentration of the produced water sample was around 20 ppm and the pH was 7.0. The quantification of sulfate-reducing bacteria from produced water samples by the MPN

method showed low microbial cell densities of 2.4 x 10^3 cells/ml. This result combined with the low sulfide concentrations measured confirmed the low activity of SRB in the produced water sample. The designed SRB culture APB55 showed the presence of SRB belonging to the family of Desulfovibrionaceae and the genus *Desulfobacter* by using FISH and SRB-385, DSV-698 and SRB-129 probes (Fig. 1).

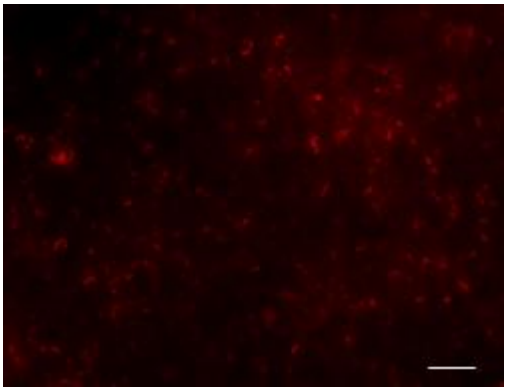

Fig. 1 Mixed culture of SRB (APB55) visualized by fluorescence in-situ hybridization (FISH) using SRB-385 probe and Cy3 as a fluorochrome. Bar represents 5 µm

Biogenic production of sulfide

Results obtained from a mixed culture of sulfate-reducing bacteria (APB55) showed that the culture produced sulfide during its growth, when CG was used at concentrations of 1 (data not shown) and 2%. The sulfide level reached more than 250 ppm for 2% CG, which equates to the complete utilization of all available sulfate in the medium. Sulfide production by APB55 with CG originating from soybeans was similar to the control (commercial glycerol) (Fig. 2A). However, the initial sulfide production was almost two times lower than compared to the commercial glycerol. All other types of CG resulted in a delayed sulfidogenic activity of the culture or lowered final concentrations of sulfide.

At concentrations of 3, 4% (data not shown) and 5% CG the biogenic production of sulfide was much lower (castor beans and soybeans-type CG) or even completely inhibited (OWF- and cotton-type CG) (Fig. 2B and 2C). At 3% CG the sulfide level was reduced from more than 250 ppm (control) to values close to 30 ppm. Only crude glycerol from soybeans reached the same end concentration of sulfide compared with the control, though having a slower start-up (Fig. 2B). Overall the inhibition of biogenic sulfide formation was most effective when using high CG concentrations (5%). Then sulfide levels were reduced to approximately 10 ppm. Only in cultures with CG from soybeans the values reached close to 50 ppm sulfide (Fig. 2C).

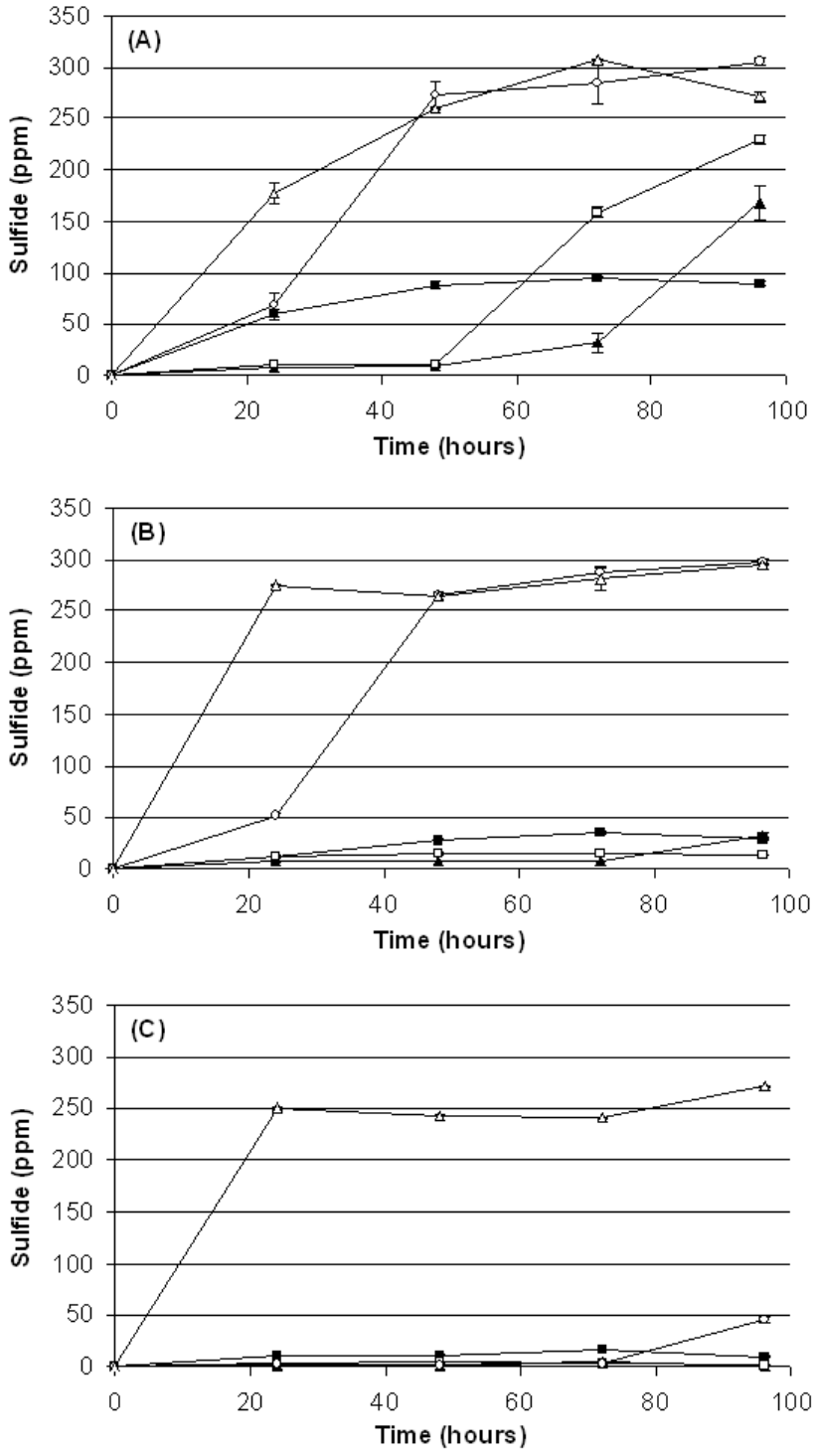

Fig. 2 Sulfide production by a mixed culture of sulfate-reducing bacteria (APB55, 10^5 cells/ml inoculum) using crude glycerol from biodiesel production plants using different source materials: ■, castor beans; ▲, cotton; **X,** oils and waste fats (OWF)**;** □, soybeans; Δ, control (pure glycerol). The concentration of the substrates varied as follows: A) 2%, B) 3% and C) 5% crude glycerol (w/v). means ± ranges (error bars), n = 2

Also the inoculum density (10^3 and 10^4 cells/ml of APB55 compared to 10^5 cells/ml) had an influence on biogenic sulfate formation (data not shown). The lower the initial cell density the

higher the inhibitive effect of CG on sulfate reduction compared to the controls with commercial glycerol.

Chemical composition of crude glycerol

The concentration of pure glycerol in the different samples of crude glycerol samples ranged from 2 to 40 mg/ml (Table 2). Especially cotton- and soybeans-type CG showed very low concentrations of glycerol. These values were below expected ones reported in literature (Hazimah et al. 2003; Silva et al. 2009). The lower glycerol content in cotton- and soybeans-type CG samples may be related to the presence of polymers of glycerol formed during the production of biodiesel (Journal of Plastic 2010). These glycerol complexes are probably not or to a lower degree reactive in the assay, underestimating the total glycerol concentration.

Table 2 Chemical composition of crude glycerol samples, deriving from biodiesel plants processing different source materials

Composition	**Crude glycerol**			
(mg/ml)	Castor beans	Cotton	OWF	Soybeans
Glycerol	20.98	2.57	41.87	2.66
Formaldehyde	0.12	-	0.05	-
Acetaldehyde	1.26	0.11	0.70	0.03
Acrolein	0.10	0.23	0.18	12.60
Propionaldehyde	-	0.63	0.19	0.10
Butiraldehyde	-	0.31	0.16	0.06
Benzaldehyde	-	0.51	-	-
Isovaleraldehyde	-	0.22	0.11	-
Valeraldehyde	-	1.70	0.52	0.21
o-tolualdehyde	-	0.17	0.15	-
m-tolualdehyde	0.20	0.58	-	0.08
p-tolualdehyde	0.04	3.38	0.83	0.29

-, not detected; OWF, stands for oils and waste fats

The quantification of the aldehydes using liquid chromatograph identified some carbonyl compounds (Table 2). Some of these compounds have earlier been reported to inhibit the growth of SRB, such as acrolein and formaldehyde (Feron et al. 1991; Ma and Harris 1988;

PPRC 1993). The concentrations of acrolein were highest in CG from soybeans, whereas formaldehyde was most enriched in CG from castor beans (Table 2).

Discussion

Crude glycerol is a cheap carbon source and could be seen as potential substrate for many biotechnological processes. Next to other applications crude glycerol has successfully been used for microbial enhanced oil recovery purposes. However, several questions have still remained open, such as which microorganisms are involved and foremost what is the impact of such a treatment on the sulfate-reducing community and consequently on souring.

The main problems of souring are the reduction of productivity and quality of produced hydrocarbons, and increased corrosive processes due to the activity of sulfate reducers (Hubert et al. 2005; Coetser and Cloete 2005). In our study SRB indigenous to oil fields were used in order to mimic the native in-situ microbiology and to have a stronger predictive power in this question. Two groups of sulfate reducers were isolated from PW and used for further experiments; they belonged to the family Desulfovibrionaceae and the genus *Desulfobacter*. Voordouw et al. (1996) observed that all SRB detected from different oil fields using the 16S rRNA gene belong to the families Desulfovibrionaceae or Desulfobacteriaceae. *Desulfovibrio* is a regularly found genus of SRB in produced waters from oil fields and several species have been isolated from this environment (Magot et al. 1992; Tardy-Jacquenod et al. 1996; Nga et al. 1996; Magot et al. 2004; Miranda-Tello et al. 2003).

The biological conversion of 1 mM glycerol to acetate theoretically reduces 0.75 mM sulfate to sulfide. However, the complete oxidation of glycerol coupled to sulfate reduction reduces 1.75 mM sulfate. More sulfide formation than stoichiometrically expected may also be related to the broad variety of compounds present in CG samples besides glycerol (such as organic acids). These compounds may also contribute as potential electron donors to the reduction of sulfate and consequently more sulfide would be formed than stoichiometrically expected from glycerol oxidation only.

In this work, crude glycerol was shown to be successfully used as electron donor for the biological reduction of sulfate to sulfide by a mixed culture of SRB, deriving from an oil field. Several sulfate-reducing bacteria are able to use glycerol as electron donor. Several *Desulfovibrio* species were reported to grow with glycerol, such as *D. fructosovorans*, *D. alcoholovorans*, *D. vulgaris*, *D. carbinolicus*, *D. giganteus* (Stams et al. 1985; Kremer and Hansen 1987; Nanninga and Gottschal 1987; Esnault et al. 1988; Ollivier et al. 1988; Qatibi et al. 1991; Ouattara et al. 1992). The ability to use glycerol was also demonstrated for

Desulfospira joergensenii, isolated from marine sediment (Finster et al. 1997) and members of the genus *Desulfosporosinus* originating from acid sediments (Alazard et al. 2010; Sánchez-Andrea et al. 2013).

However the study also shows that concentrations of more than 3% crude glycerol greatly inhibit the biogenic production of sulfide. This could possibly be related to the presence of toxic compounds in CG. Chromatographical analyses of CG samples identified aldehydes that may be associated with an inhibition of microorganisms, as has been shown for acrolein and formaldehyde (Feron et al. 1991; Ma and Harris 1988) (Table 2). Acrolein is the main product of dehydration of glycerol during transesterification processes. It is also found in biofuels plants when fats and oils are directly used in catalytic thermolysis reactions (Parshall and Ittel 1992). Formaldehyde is the main product formed by the catalytic oxidation of methanol (Allison and Goddard III 1984; Reuss at al. 2002), which is usually used in the transesterification process. In this way, both acrolein and formaldehyde can be formed during the biodiesel production. These compounds are also used in biocides to control the growth of gram-positive and gram-negative bacteria, fungi and yeast (Feron et al. 1991; Ma and Harris 1988; PPRC 1993). Nevertheless, in this study we did not observe a direct relationship between the measured concentrations of acrolein and formaldehyde, and the inhibition of sulfidogenesis. The most inhibitive effect on the formation of hydrogen sulfide was observed with CG samples deriving from OWF and cotton utilizing biodiesel plants (Fig. 2B/C). The highest concentrations of acrolein (in CG from soybeans) and formaldehyde (in CG from castor beans) were found for CG samples that were less inhibitive for sulfidogenesis (Table 2 and Fig. 2B/C).

Alkali metals are important for the cultivation of bacteria, but they can also inhibit the growth of microorganisms when present in high concentrations. The inhibitory concentration of sodium ion (Na^+) is between 3 to 16 g/l (Feijoo et al. 1995) and potassium ion (K^+) is 2 to 12 g/l (Bashir and Matin 2004). Chaves et al. (2010) detected high values of sodium (0.36 to 19 g/l) and potassium (<0.08 to 92 g/l) in CG samples from vegetable waste oil and commercial soybeans oil. The authors also observed that there is a large variation in the concentrations of Na^+ and K^+ in the CG samples. This indicates that much of the catalyst used is concentrated in the crude glycerol phase. The high concentration of Na^+ and K^+ may be related to the choice of catalyst used in the biodiesel production, which are usually sodium hydroxide (NaOH) or potassium hydroxide (KOH).

We suppose that waste compounds from biodiesel production, such as particular carbon compounds (acrolein and formaldehyde) and the high concentration of catalysts (NaOH or

KOH) in CG act directly or via an associated way on the metabolism of SRB. Further studies have to be conducted for a better understanding of the inhibitory effect of higher concentrations of CG on the biogenic formation of sulfide.

A definite prediction about the potential of sulfide formation after CG injection in oil fields is certainly not possible from this study. However the results show that CG is utilized by indigenous SRB and may therefore contribute to an increase of souring in oil reservoirs. A continuous dosing of sufficiently high CG concentrations to inhibit sulfidogenesis seems impracticable and would not be maintainable throughout an oil reservoir.

However, the study on the other hand suggests CG as a good electron donor for industrial processes. Combined with the market price and the large supply of CG it might for instance be an ideal candidate for metal precipitation processes based on biologically formed sulfide.

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CAPÍTULO 3: ENRICHMENT OF BACTERIA WITH GLYCEROL AND SULFATE FROM ANAEROBIC BIOREACTOR SLUDGES AND TINTO RIVER SEDIMENTS

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Abstract Glycerol is a main co-product of biodiesel production. Crude glycerol is a cheap and attractive substrate for biotechnological application, such as growth of sulfate-reducing bacteria to produce sulfide for metal precipitation. In this work, sulfate reduction with glycerol was studied at neutral and acidic pH using two sludge samples from bioreactors and Tinto River sediment, respectively, as inoculum. Sulfate-reducing bacteria and fermentative bacteria were co-enriched. Molecular analysis revealed that *Desulfomicrobium* was dominant in the cultures enriched at pH 7, while *Desulfosporosinus* and bacteria modestly related to *Propionispora* and *Paludibacter* were dominant in the culture enriched at pH 4. Glycerol conversion was coupled to sulfate reduction, but the substrate was incompletely oxidized to acetate in the neutrophilic enrichments, while in the acidic enrichment acetate, lactate and 1.3-propanediol were detected. Two strains belonging to *Desulfomicrobium* and *Clostridium* genera were isolated from the neutrophilic enrichments, but these isolates were not able to use glycerol. A *Clostridium* strain able to grow with glycerol was isolated from the low pH enrichment. Our data indicate that glycerol promotes growth of sulfate-reducing communities to form sulfide, which might be used to precipitate and recover heavy metals.

Keywords Glycerol, Sulfate-reducing bacteria, Fermentative bacteria, Metal recovery

Introduction

Biodiesel is a biofuel that is produced by the transesterification of vegetable oils or animal fats with ethanol or methanol, resulting in the formation of crude glycerol as a main by-product (Silva et al. 2009). The massive production of biodiesel has created opportunities for biotechnological application of glycerol as its market price has dropped considerably in the past decade (Yazdani and Gonzalez 2007). Currently, crude glycerol is considered as a waste product due to the presence of impurities such as alcohol, salts and heavy metals (Johnson and Taconi 2007). Innovative processes to apply glycerol are under development, such as conversion of glycerol to propylene glycol and acetone (Chiu et al., 2006; Dasari et al. 2005) or use of crude glycerol as fluid for oil recovery in the micro-reservoir cell that simulated the oil well conditions (Quintella et al. 2006).

Several microorganisms are able to grow anaerobically with glycerol as the sole carbon and energy source (Seifert et al. 2001; Biebl et al. 1998, 1999). Microbial glycerol fermentation is an interesting way to produce valuable compounds such as 1,3-propanediol (PDO), acetic acid, butyric acid, acetone, ethanol, 2,3-butanediol, lactic acid, succinic acid, formate, and H_2 (Biebl et al. 1999; Ito et al. 2005; Zeng 1996; Zeng and Biebl 2002). Glycerol fermentation has been observed in species belonging to the genera *Klebsiella*, *Citrobacter*, *Enterobacter*, *Clostridium, Propionibacterium* and *Trichococcus* (Johnson and Taconi 2007; Biebl at al. 1999; Himmi et al. 2000; van Gelder et al. 2012). Glycerol may also be used as electron donor for reduction processes such as sulfate reduction. Sulfate reduction is a process that can be applied to the removal of heavy metals from waste streams (Hulshoff Pol et al. 2001). Sulfate-reducing bacteria occur in anoxic habitats, where they reduce sulfate to sulfide with organic compounds or hydrogen as electron donor (Muyzer and Stams 2008; Postgate 1984). Several *Desulfovibrio* species were reported to grow with glycerol (Stams et al. 1985; Kremer and Hansen 1987; Nanninga and Gottschal 1987; Esnault et al. 1988; Ollivier et al. 1988; Qatibi et al. 1991; Ouattara et al. 1992). In the presence of sulfate, *D. carbinolicus* and *D. fructosovorans* oxidize glycerol to 3-hydroxypropionate and acetate, respectively, while both strains ferment glycerol to 1,3-propanediol and 3-hydroxypropionate in the absence of sulfate (Nanninga and Gottschal 1987; Ollivier et al. 1998). The ability to use glycerol was also shown for members of the acidotolerant genus *Desulfosporosinus* (Alazard et al. 2010; Sánchez-Andrea et al. 2013). The aim of this study was to investigate the potential of glycerol as electron donor for sulfate reduction, using neutrophilic and acidophilic inocula as sources.

Materials and Methods

Inocula

Three different inocula were used for the enrichments; two neutrophilic sludges samples from bioreactors and a sulfate-reducing enrichment with low pH sediment (Tinto River). The neutrophilic sludges were obtained from anaerobic bioreactors for biological sulfate reduction to produce sulfide for metal removal. One sludge originated from a chemical plant in Emmen the Netherlands (Dar et al 2007) and the other sludge from a zinc smelter in Budel, The Netherlands (Sipma et al 2003). These sludges were termed Paques sludge and Budel sludge, respectively. Sludges were crushed as described by Oude Elferink et al. (1995). The third sample was a mixture of acidic sediment that was collected from Tinto River Basin (Huelva) in September 2011 at two sampling sites: JL (37.691207°N, 6.560587°W) and SN (37.72173°N, 6.557465°W) dams. Detailed information of the sampling sites was presented recently (Sánchez-Andrea et al. 2012).

Medium composition and growth conditions

The basal medium (Stams et al. 1993) that was used for enrichment contained the following (g/L): 0.53 $Na_2HPO_4{\cdot}2H_20$, 0.41 KH_2PO_4, 0.3 NH_4Cl, 0.11 $CaCl_2{\cdot}2H_2O$, 0.1 $MgCl_2{\cdot}6H_20$, 0.3 NaCl and 0.48 $Na_2S{\cdot}9H_2O$, as well as the acid and alkaline trace elements (1 mL/L each) and vitamins (0.2 mL/L). The basal medium was supplemented with 0.1 g/L yeast extract (BBL, Becton Dickinson, Cockeysville, Md.) and 1.42 g/L Na_2SO_4. For the neutral pH enrichment 4 g/L $NaHCO_3$ was added as a buffer. The pH of the Na_2S solution was adjusted to pH 5 with HCl (37 %) prior to addition to the vials. pH 7 and 4 were used for neutral and acidic enrichments, and the proton concentration in the medium was adjusted by using 2N NaOH or 2N HCl. Sulfate (20 mM) and glycerol (20 mM) were added as electron acceptor and donor, respectively. All compounds were heat-sterilized at 121ºC for 20 min except for the vitamins and the Na_2S solution, which were filter-sterilized. The 120-ml serum bottles filled with 50 ml medium were sealed with butyl rubber stoppers (Rubber BV, Hilversum). The gas phase was 1.5 atm N_2/CO_2 (80:20, v/v). The medium was sterilized at 121°C for 20 min. Primary neutrophilic and acidophilic enrichments were incubated statically at 30ºC for 11 and 35 days, respectively.

Isolation of bacteria

To obtain axenic cultures, serial dilutions from primary enrichments were made in fresh medium. Bacteria were isolated from the highest dilution (10^{-8} and 10^{-9} cells/mL) with growth

by spreading 0.1 mL of diluted samples on agar plates (agar 1.5 %, w/v) prepared with medium that contained glycerol as substrate and sulfate as electron acceptor. These handlings were done in an anaerobic chamber and the plates were incubated in anaerobic jars as described by Watt et al. (1976). One percent of Wilkins-Chalgren anaerobe broth (CM0643, Thermo Scientific), containing tryptone (10 g/L), gelatin peptone (10 g/L), yeast extract (5 g/L), glucose (1 g/L) and sodium pyruvate (1 g/L) as a typical formula, was also used as substrate to isolate fermentative bacteria. Individual colonies that became visible between 3 to 7 days of incubation were streaked on new plates again. The streaking procedure was repeated two times more. Finally, single colonies were picked up and inoculated into serum bottles. Purity of the cultures was checked by microscopic examination after growth and by molecular biological analyses. A phase contrast microscope (Leica DM 2000) equipped with a digital camera (Leica DFC 420) was used to examine the morphology of cultures.

Bacterial strains

Desulfomicrobium baculatum (DSM 4028^T) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. Bacteria were first cultured in medium number 63 (DSMZ) and transferred into serum bottles containing basal medium with glycerol (20 mM) as substrate and sulfate (20 mM) as electron acceptor. This strain was also tested for hydrogen utilization as electron donor in vials flushed with H_2/CO_2 as headspace.

Analytical methods

Sulfate reduction activity of cultures was monitored by measuring sulfide colorimetrically with the methylene blue method (Trüper and Schlegel 1964). Bacterial growth was assessed by measuring the optical densities at 600 nm (OD_{600}). Organic compounds were analysed by high-pressure liquid chromatography (SpectraSystem, Thermo, country) equipped with a Varian Metacarb 67H column (300 x 6.5 mm) kept at 30°C and RI refractive index detector. The mobile phase was 0.005 M sulfuric acid as eluent at a flow rate of 0.8 mL/min. Gasses were measured with a Gas Chromatograph (Shimadzu GC-2014) equipped with a Molsieve 13x column and hold at 100°C. Injections were direct on column via an injection block held at 80°C. The carrier gas was Argon with a flow of 50 mL/min. The detector was a TCD detector, hold at 130°C with a current of 70 mA. For the quantification of sulfate, an ion chromatograph (Model ICS-1500, Dionex, Sunnyvale, CA, USA) equipped with IonPac AS22 analytical column (2 x 250 and 4 x 250 mm) and Ed40 detector was used. The eluent was a

mixture of 0.45 M sodium carbonate and 0.14 M sodium bicarbonate and the eluent flow-rate was set at 1.0 mL/min. All the instruments control and data collection were performed with ChromQuest 5.0 software (Dionex, USA).

DNA extraction and PCR amplification

Total DNA from enrichments and pure cultures were extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, USA) according to the manufacture's protocol. Polymerase chain reaction (PCR) amplifications were performed using the isolated DNA and specific primers (Table 1) targeting the bacterial and archaeal 16S rRNA gene. PCR products were checked by electrophoresis on a 1 % agarose gel (w/v). Gel was stained with Sybr® Safe (Invitrogen, USA) and visualized on a gel-doc system (Bio-Rad, USA).

Table 1 Primers used in this study for 16S rRNA gene PCR amplification.

Primer	Sequence (5`- 3`)	Specificity	Reference
Bact27f	GTT TGA TCC TGG CTC AG	16S rRNA library construction	Weisberg et al. 1991
Arch109f	ACK GCT CAG TAA CAC GT	16S rRNA library construction	Grosskopf et al. 1998
Uni1492r	GGT TAC CTT GTT ACG ACT T	16S rRNA library construction	Weisberg et al. 1991
968f-GC	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC	DGGE	Nübel et al. 1996
1401r	CGG TGT GTA CAA GAC CC	DGGE	Nübel et al. 1996
Sp6	GAT TTA GGT GAC ACT ATA G	Sequencing	Promega
T7	TAA TAC GAC TCA CTA TAG GG	Sequencing	Promega

DGGE analysis

Extracted DNA from enriched and pure cultures was amplified by PCR using a primer set that targets the V6-V8 regions of the 16S rRNA (Table 1). Forward primer had attached to its 5' end a GC clamp (Table 1). PCR products were analysed by Denaturating Gradient Gel Electrophoresis (DGGE) using a Dcode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) (Muyzer et al. 1993). DGGE was performed on polyacrylamide gels

with a denaturant gradient from 30 % to 60 % for the separation of 16S rRNA gene amplicons (100 % denaturing acrylamide was defined as 7 M urea and 40 %, v/v, formamide). Aliquots of PCR products were loaded on the gel and electrophoresis was carried out with 0.5 X Tris-acetate-EDTA buffer at 60ºC and at 85 V for 16 h. After the completion of the electrophoresis, gels were silver-stained (Sanguinetti et al. 1994) and scanned. DGGE band detection and quantification of band intensity were performed using the Bionumerics software version 4.61 (Applied Maths, Belgium) (Tzeneva et al. 2009). A dendrogram was created using Unweighted Pair Group Analysis (UPGMA) and dice similarity coefficient on Bionumerics software.

Cloning and sequencing of microbial 16S rRNA

16S rRNA gene fragments previously amplified by PCR using the primer pairs Bact27-f and Uni1492-r (Table 1) were purified using the High Pure PCR Cleanup Micro kit (Roche Applied Science, Almere, Netherlands). PCR products were cloned in *Escherichia coli* DH5α competent cells by using the pGEM-T vector (Promega, Madison, WI) according to the manufacturer's instructions. Individual colonies were screened by PCR using the primers Sp6 and T7 (Table 1). PCR products were sequenced with primers mentioned above at GATC Biotech (Konstanz, Germany).

Phylogenetic analysis of 16S rRNA gene sequences

Sequences were assembled using the DNABaser (v3.4.3) program, and prior to phylogenetic analysis, vector sequences flanking the 16S rRNA gene inserts were removed. Similarity searches were performed using the BLAST search program (Altschul et al. 1990) and the Ribosomal Database Project (Maidak et al. 2001) to identify the closest sequences. Clone sequences were checked for chimeras using the program Chimera Check from green genes (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi). A total of 64 complete sequences (>1400 bp) obtained in this study were added to a database of over 50000 homologous prokaryotic 16S rRNA primary structures by using the alignment tool of the ARB software package. Phylogenetic reconstruction was performed by using the three algorithms as implemented in the ARB package (Ludwig et al. 2004). A consensus tree was generated and bootstrap analysis was performed.

Nucleotide sequence accession numbers

Sequences of the 16S rRNA gene clones and isolated bacteria were deposited in the GenBank database under accession numbers: KC215456 to KC215464.

Results

Enrichment of bacteria with glycerol and sulfate

Bacteria were enriched at pH 4 and pH 7 from acidic mixed sediment samples and two neutrophilic sludges with glycerol and sulfate. The cultures were obtained by repeated transfers of full-grown cultures to the same fresh medium. Routinely, when using an inoculum size of 1 % full-grown cultures were obtained in about 10 and 30 days for neutral and acid enrichments, respectively.

Upon microscopic observation the neutrophilic enrichments consisted of different kinds of rod-shaped cells that formed spores (Fig. 1). The Tinto River enrichments also contained different forms of cells (Fig. 1). In one of the acidophilic enrichments (sample 1) three types of rod-shaped cells were visible, while in another enrichment (sample 2) mainly contained slightly curved cells that formed spores. Both samples were duplicates with same inoculum and culture conditions.

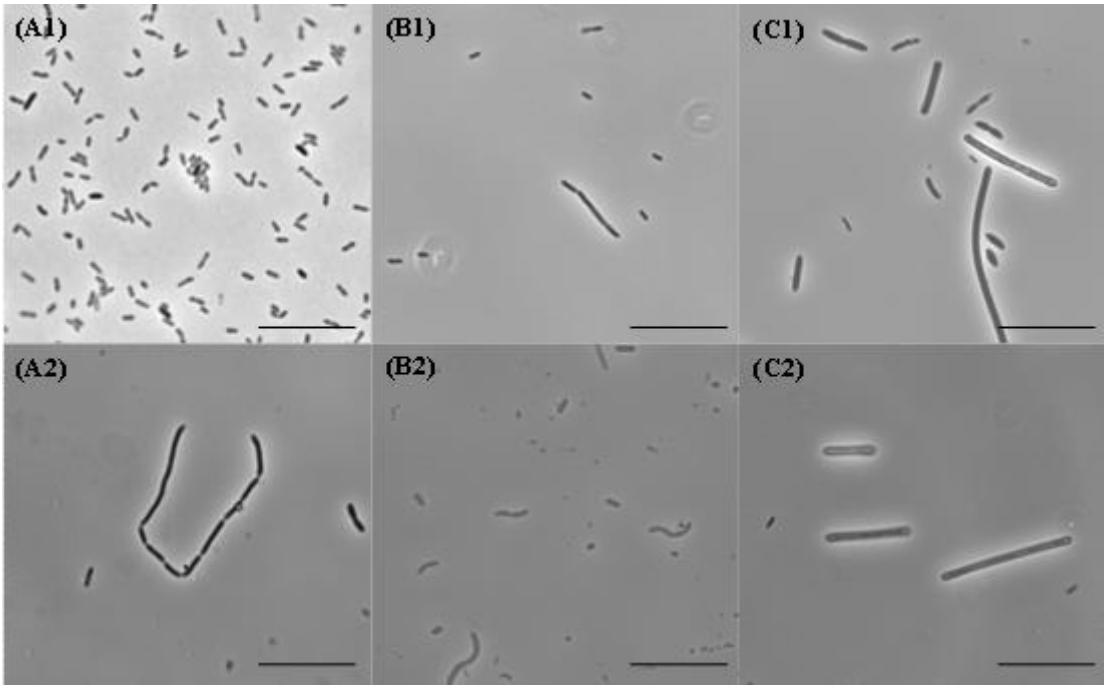

Fig. 1 Cell morphology of bacteria from Paques (A), Budel (B) and Tinto River (C) enrichments with glycerol and sulfate (1), and without sulfate (2). Bar represents 10 µm

Enrichments from Budel and Paques reached OD_{600} values of 0.11 to 0.17 in the presence of sulfate. When enrichment was continued in the absence of sulfate growth was still observed, but the OD_{600} values were only 0.03 to 0.10. The pH in the incubation remained between 7.0 and 7.4. The main products formed from microbial conversion of glycerol by Budel and Paques enrichments in presence of sulfate were acetate and sulfide, and in absence of sulfate acetate, formate and H_2 were formed (Table 2). Methane was not formed in the enrichment cultures, neither in the presence nor the absence of sulfate.

Table 2 Main products formed by glycerol (20 mM) degradation in enrichments in the presence (20 mM) or absence of sulfate.

Enrichments	Mean end products from glycerol (mM)						Sulfide
	Glycerol conversion	lactate	acetate	formate	1.3-PDO	H_2	(mM)
Tinto River	15.1	1.6	1.1	-	4.5	-	2.5
	7.7	-	-	-	4.8	2.7	na
Paques	16.0	-	13.5	-	-	-	3.8
	10.9	-	4.2	2.5	-	16.6	na
Budel	11.9	-	9.0	-	-	-	3.3

-, not detected; na, not applicable as no sulfate was present.

The enrichment with sulfate from Tinto River reached OD_{600} values of about 0.25. Growth was associated with a pH increase from pH 4 to pH 5.5 and acetate, lactate, 1.3-propanediol (1.3-PDO) and sulfide were formed as main products. Omission of sulfate resulted in growth, but the OD_{600} was just about 0.10 and no change in pH was observed. In the absence of sulfate, glycerol was converted to 1.3-PDO and H_2 (Table 2). Methane was also not formed in any of the acidophilic enrichments.

Community analysis of the enrichments

With archaeal primers, no PCR products were obtained, while amplification products were obtained with a control archaeon, *Archaeoglobus fulgidus* VC-16, suggesting that Archaea were not enriched under these conditions. This is consistent with the observation that methane was not formed in any of the enrichments.

To get insight into which bacteria were involved in the conversion of glycerol with sulfate, clone libraries were made. From the 19 clones of the Paques enrichment, 17 belonged to a sulfate-reducer closely related to *Desulfomicrobium norvegicum* and *D. baculatum* (99% sequence similarity). All the 12 clones of the Budel enrichment also belonged to *Desulfomicrobium* (Table 3). As seen in Fig. 2, there is a high phylogenetic affiliation of the sequences from neutral enrichments. DGGE analysis of the enriched cultures showed two dominant bands in both Budel and Paques enrichments (Fig. 3), confirming the microscopic observation.

Table 3 Sequences, number of clones, accession number and phylogenetic affiliation from enrichments

Sequences	Clones		*Phylogenetic Affiliation	Similarity
	Number	Accession No.	Accession No.	(%)
Clone from Paques	17	KC215457	*Desulfomicrobium norvegicum*	99
			Desulfomicrobium baculatum	99
Clone from Budel	12	KC215456	*Desulfomicrobium norvegicum*	99
			Desulfomicrobium baculatum	99
Clone from Tinto River (sample 1)	25	JQ433965	*Desulfosporosinus acidiphilus*	97
	3	KC215460	*Porphyromonadaceae* bacterium	93
			Paludibacter propionicigenes	89
Clone from Tinto River (sample 2)	5	KC215459	*Propionispora hippei*	93

*Similarity to nearest neighbor in the GenBank nucleotide database as determined by BLAST results;

From one of the Tinto River enrichments 33 clones were obtained of which 25 had 97 % sequence identity with *Desulfosporosinus acidiphilus* (Table 3). Eight other clones were distantly (89% and 93 % sequence similarity, respectively) related to *Paludibacter* and *Propionispora* (Table 3). The phylogenetic tree based on 16S rRNA genes is shown in Fig. 2 and the DGGE analysis in Fig. 3.

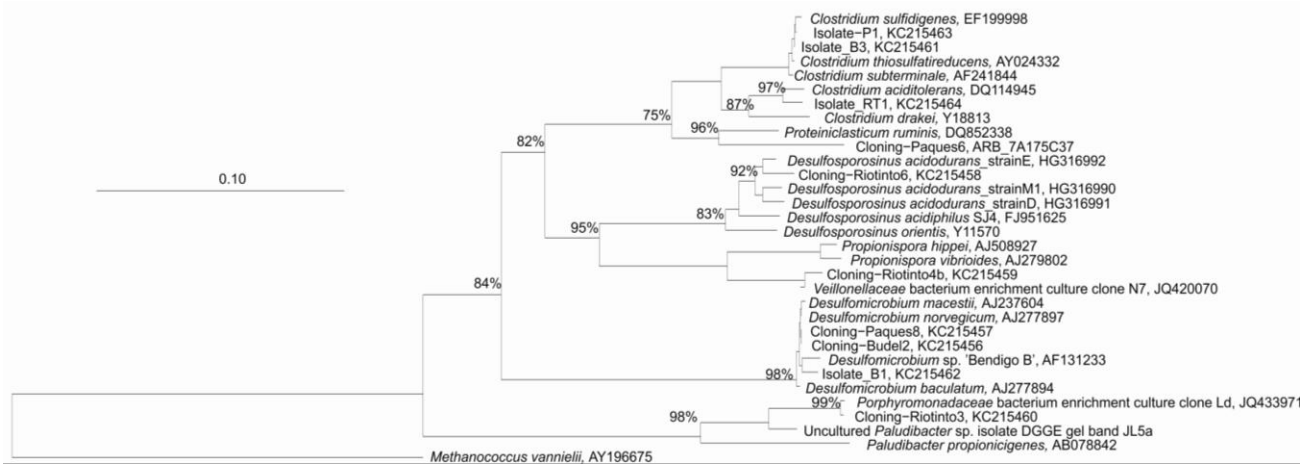

Fig. 2 DGGE profiles of bacterial PCR-amplified 16S rRNA gene fragments obtained from enrichments and pure cultures. A, B3 isolated from Budel enrichment; B, B1 isolated from Budel enrichment; C, P1 isolated from Paques enrichment; D, R1 enrichment from Tinto River enrichment; E, marker; F, enrichment from Budel; G, enrichment from Paques; H and I, enrichment from Tinto River (samples 2 and 1, respectively)

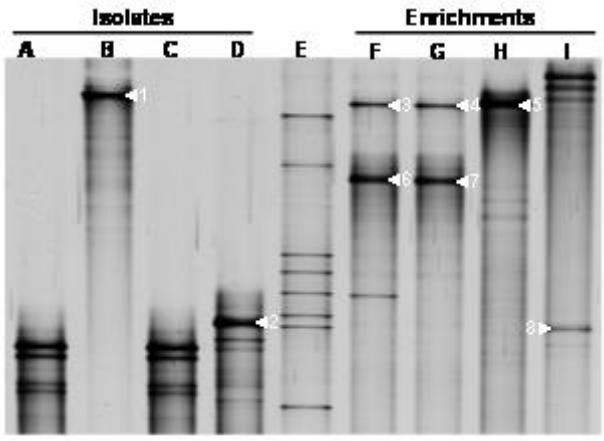

Fig. 3 Phylogenetic tree of bacterial 16S rRNA gene sequences from Budel, Paques, Tinto River enrichments and pure cultures was generated using parsimony with the ARB program. The bar indicates a 10% estimated sequence divergence. The sequences are indicated in boldface type, and the designations after the organism names or identifiers are GenBank accession numbers

Isolation of strains

Attempts were made to isolate glycerol-degrading sulfate-reducing bacteriafrom the Budel and Paques enrichments. Colonies were formed when enrichments were plated on media with glycerol and sulfate. However, the colonies that were obtained always consisted of two bacteria with the morphologies that were observed in the enrichments. Using Wilkins-Chalgren as substrate a sulfate-reducer (strain B1) was isolated from the sulfate-reducing Budel enrichment. The 16S rRNA sequence of strain B1 (Accession No. KC215462) had high similarity (99 %) with two *Desulfomicrobium* species, *D. baculatum* and *D. norvegicum*

(Table 3 and Fig. 2). These two bacteria are non-spore-forming, Gram-negative, sulfate-reducing and obligately anaerobic rods (Genthner at al. 1997; Azabou et al. 2007). Isolate B1 cells were rod-shaped (0.5 - 1 µm x 3 - 5 µm) with rounded ends and occurred singly or in pairs, motile and non-spore forming (Fig. 4a). Although strain B1 was isolated from an enrichment with glycerol and sulfate, it did not use glycerol as electron donor for sulfate reduction. The closely related *D. baculatum* (DSM 4028^T) was also tested, but it was not able to use glycerol. This strain as well as our strain B1 are capable to use lactate, pyruvate and H_2 coupled to the reduction of sulfate.

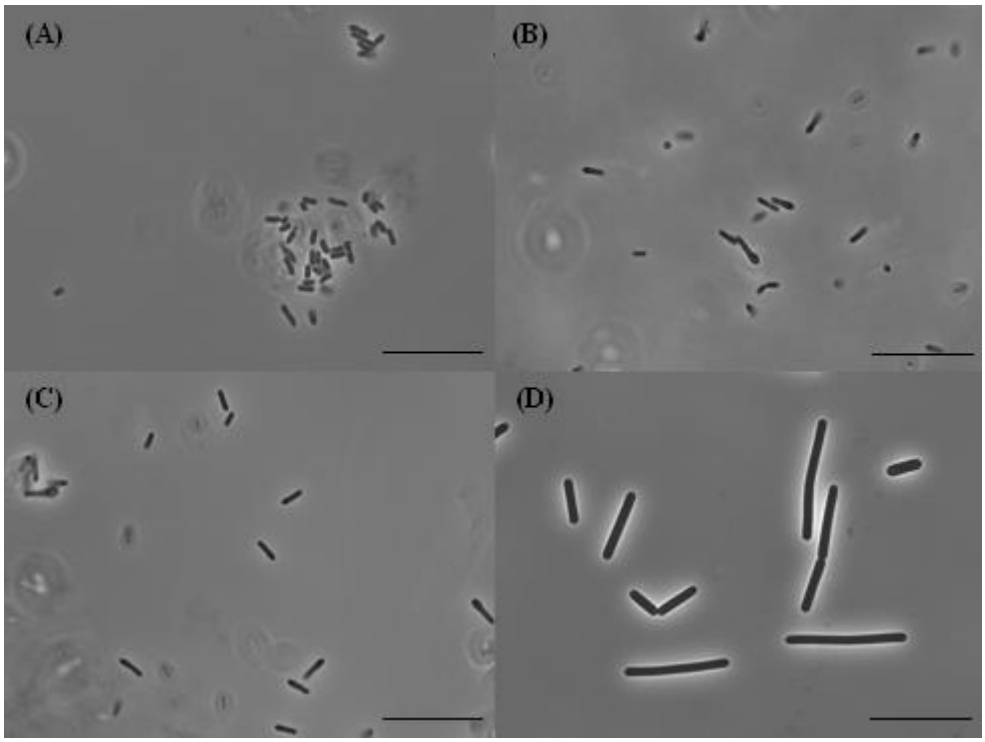

Fig. 4 Cell morphology of B1 (A), B3 (B), P1 (C) and TR1 (D) isolates from Budel, Paques and Tinto River enrichments, respectively. Bar represents 10 µm

Using Wilkins-Chalgren as substrate also two spore-forming bacteria were isolated (strain B3 and P1) from the Budel and Paques enrichments, respectively. The two strains showed similar cell morphologies. The cells were rod-shaped (0.5 - 1 µm x 2 - 6 µm) and occurred singly and in pairs. Terminal spores were formed (Fig. 4b and 4c). Strain B3 (Accession No. KC215461) and strain P1 (Accession No. KC215463) had an identical rRNA gene sequence and belonged to clostridial cluster I. The sequence was related to *Clostridium thiosulfatireducens* and *Clostridium sulfidigenes* (99% similarity), bacteria that have highly similar rRNA gene sequences. For none of the described bacteria the ability to use glycerol has been tested so far

(Hernández-Eugenio et al. 2002; Sallam and Steinbüchel 2009). Strains B3 and P1 were tested for the ability to grow with glycerol, but glycerol was not used. We made co-cultures of the sulfate-reducing *Desulfomicrobium* strain B1 and the *Clostridium* strains B3 and P1. These cocultures were also not able to use glycerol. Strains B3 and P1 were not dominantly present in the enrichments cultures with glycerol and sulfate (Fig. 3). Unfortunately, despite some attempts the dominant *Clostridium* in the enrichments could not be isolated.

Strain TR1 was isolated from the Tinto River enrichments with Wilkins-Chalgren as substrate. The rRNA gene sequence (Accession No. KC215464) showed high similarity (98 %) with *Clostridium aciditolerans* (Fig. 2). *C. aciditolerans* cells are straight to slightly curved rods, 0.5 - 1 µm in diameter and 3 - 9 µm in length. Spores are subterminal and oval in shape and do not swell the cell. Tryptone, peptone, pyruvate, glucose are used as substrates, and glucose is fermented to acetate, butyrate and ethanol as end products (Lee et al. 2007). Isolate TR1 cells were straight to slightly curved rods (1 - 1.5 µm x 3 - 15 µm) and occurred singly, in pairs or short chains. Spores are also subterminal and oval in shape (Fig. 4d). When transferred to media with glycerol, growth was observed. Like *C. aciditolerans* strain TR1 forms acetate and butyrate as products, but it did not form the typical products that we detected in the low pH enrichment cultures. No data are available regarding the potential glycerol utilization by *C. aciditolerans.* Sulfate is not used by strain TR1 and *C. aciditolerans* either (Lee et al. 2007). Bands in the DGGE from strain TR1 and Tinto River enrichment (Sample 1) were at the same position (Fig. 3).

Discussion

Glycerol as electron donor for sulfate reduction

Glycerol, a cheap and readily available by-product of biodiesel production is an electron donor for the biological reduction of sulfate to sulfide. The sulfate reduction ability can be exploited to immobilize heavy metals in sediments or to recover heavy metals in metallurgical and mining industry (Muyzer and Stams 2008). Biogenic production of sulfide has been proposed as an economic and environmentally friendly alternative for the treatment of areas impacted by heavy metals (Gibert et al. 2004; Icgen and Harrison 2006), like acid mine drainage. We investigated glycerol as electron donor for sulfate reduction at neutral and acid pH. Sulfate-reducing communities were enriched with glycerol as electron donor, but glycerol was not completely degraded coupled to sulfate reduction. By using 20 mM glycerol theoretically 15 mM sulfate can be reduced when glycerol is converted to acetate, while 35 mM sulfate can be reduced when glycerol is completely oxidized to CO_2. In our study, only

up to 4 mM of sulfide was formed from degradation of approximately 15 mM of glycerol. In the enrichments glycerol conversion lead to the formation of organic compounds, which apparently were not degraded further. This indicates that additional procedures are needed to get complete glycerol degradation to CO_2 by selecting for sulfate reducers that can degrade the organic compounds formed. It is known that acetate is a poor electron donor for sulfate reduction and that the growth rate of acetate-degrading sulfate-reducing bacteria is very low. Likely, batch enrichment is not the optimal way to get complete conversion of glycerol coupled to sulfate reduction. Rather enrichment in continuous flow systems that are monitored for low acetate concentrations are more ideal due to the toxicity of organic acids at low pH.

Bacteria in the neutral pH enrichments

In our neutrophilic enrichments *Desulfomicrobium* was dominant. The clones obtained from the enrichments at neutral pH (Budel and Paques) exhibited high 16S rRNA sequence identity (99%) to two sulfate-reducing species, *Desulfomicrobium baculatum* and *Desulfomicrobium norvegicum* (Genthner at al. 1997; Azabou et al. 2007). *D. baculatum* differs from *D. norvegicum* in its chemoautotrophic growth on H_2/CO_2 (Azabou et al. 2007). The genome from *D. baculatum* (DSM 4028) has genes coding for enzymes of the glycerol metabolism, namely glycerol kinase and glycerol-3-phosphate dehydrogenase (ecocyc.org; img.jgi.doe.gov). However, this strain as well as our isolated *Desulfomicrobium* strain did not use glycerol as electron donor for sulfate reduction. Likely, in the enrichments glycerol was used by fermentative bacteria that formed products that could be utilized by sulfate-reducing bacteria. In this work, hydrogen and formate were detected in the incubations without sulfate. These are excellent electron donors for sulfate reducers such as *Desulfovibrio* and *Desulfomicrobium* species, and they are key components in syntrophic degradation of organic compounds (Stams and Plugge 2009). Cayol et al. (2002) showed that glycerol in saline environments can be oxidized via interspecies hydrogen transfer by communities of a sulfate-reducer, *Desulfohalobium retbaense*, and two fermentative bacteria, *Halanaerobium saccharolytica* subsp. *senegalense* and *Halanaerobium* sp. strain FR1H. Most likely, interspecies hydrogen transfer has played a role in our neutrophilic enrichments. With glycerol as substrate we only obtained colonies that consisted of bacteria with different morphologies, but we failed to obtain pure cultures. Two *Clostridium* strains were isolated with Wilkins-Chalgren broth as substrate, but these strains were not abundant in the glycerol-degrading enrichments. In addition, these bacteria did not grow with glycerol, neither in pure

culture nor in co-culture with the isolated *Desulfomicrobium* species (strain B1). Our isolation strategy did not take the possibility of a syntrophic type of conversion into consideration.

Bacteria in the acidic pH enrichments

In previous studies sulfate-reducing bacteria were isolated from acidic mine streams and lakes, but these bacteria appeared to be neutrophilic and were not active below pH 5 (Küsel et al. 2001; Lee et al. 2009). Pure cultures obtained from mixed cultures capable of reducing sulfate at pH 3 were not able to reduce sulfate below pH 5.5 (Gyure et al. 1990; Tuttle et al. 1969). However, true acidophilic sulfate-reducing bacteria belonging to the *Desulfosporosinus* genus have been described (Karnachuk et al. 2009; Alazard et al. 2010; Sánchez-Andrea et al. 2013). The use of non-acidic organic substrates such as glycerol is essential for the successful enrichments of acidophilic sulfate-reducing bacteria (Johnson et al. 2009; Karnachuk et al. 2009). Almost 76% of the sequences from Tinto River clone library showed high similarity to *Desulfosporosinus* enrichment culture clone Db. (Accession No. JQ433956) from Tinto River sediments (Sánchez-Andrea et al. 2013), and *Desulfosporosinus acidiphilus* (Alazard et al. 2010) (Table 3). *D. acidiphilus* is obligatory anaerobic, spore-forming, Gram-negative, and uses glycerol as electron donor for sulfate reduction (Alazard et al. 2010; Sánchez-Andrea et al. 2013). The other two detected bacteria have low 16S rRNA gene sequence similarity with *Propionispora hippei* (93 % similarity) and *Paludibacter propionicigenes* (89 % similarity). *Propionispora hippei* uses glycerol as substrate (Abou-Zeid et al. 2004). *Paludibacter propionicigenes* does not use glycerol as substrate (Ueki et al. 2006). Both bacteria form propionate and acetate as major fermentation products from glucose (Berrios-Rivera et al. 2003; Boumba et al. 2008), but in our enrichments propionate was not detected as product of glycerol fermentation. Currently, the role of the detected bacteria in glycerol degradation is unknown. In this study, a *Clostridium* strain was isolated and it was able to use glycerol. However, that bacterium did not appear in the clone library, and it formed butyrate, which was not detected as product in the enrichment cultures.

Interestingly, in the enrichments without sulfate only 1.3-PDO and hydrogen were detected as products, but to produce these products from glycerol an oxidized compound needs to be formed. As we were not able to detect any other organic compound, it seems that bacteria with the ability to convert glycerol completely to CO_2 or that degrade acetate to CO_2 are present in the enrichment. Syntrophic acetate conversion was suggested in glycerol-degrading acidophilic cultures (Kimura et al. 2006).

Concluding remarks

In our studies glycerol was incompletely degraded and communities of sulfate-reducing bacteria and fermentative bacteria were co-enriched under neutral and acidic pH. The isolation of glycerol-degrading sulfate-reducing bacteria and fermentative bacteria from the neutral pH enrichments failed. Further research is needed to clarify if this is due to obligate syntrophic growth of the fermentative and sulfate-reducing bacteria or if we did not apply the appropriate culture conditions to obtain the glycerol-degrading bacteria. By contrast, from Tinto River sediments glycerol-degrading bacteria distantly related to *Desulfosporosinus acidiphilus* and *Propionispora hippei* were obtained previously (Sánchez-Andrea et al. 2013) and in this study a glycerol-fermenting *Clostridium* strain was isolated.

Glycerol is an interesting and cheap electron donor to stimulate growth of sulfate-reducing bacteria in different environments. Microbial conversion of glycerol in reduction processes could have a potential biotechnological application in metals precipitation processes. However, further research efforts are needed to use glycerol more efficiently when coupled to sulfate reduction.

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Conclusão Geral

As amostras de glicerina bruta foram usadas como substrato pela cultura mista de bactérias redutoras de sulfato (APB55) nas concentrações abaixo de 3%, tornando-se uma fonte de carbono alternativa em processos de redução biológica de sulfato. Entretanto, nas concentrações a partir de 3%, a GB mostrou atividade para inibir a produção de sulfeto, sendo as impurezas presentes na GB, tais como compostos carbônicos (acroleína e formaldeído) e a alta concentração de catalizadores (NaOH ou KOH) os principais suspeitos de atuarem de forma direta ou associada sob o metabolismo das BRS. Nesse sentido, mais estudos devem ser conduzidos para compreender o efeito inibitório da GB em altas concentrações sob a formação biológica de sulfeto.

O enriquecimento de solos anóxicos indicou que a glicerina comercial foi incompletamente degradada e que as comunidades de bactérias redutoras de sulfato e bactérias fermentativas foram co-enriquecidas em pH ácido e neutro. Não foi possível isolar BRS oxidante de glicerina e bactérias fermentativas dos enriquecimentos de pH neutro. Novas pesquisas são necessárias para esclarecer se isto foi devido à relação sintrófica de crescimento entre as bactérias fermentativas e as BRS ou se não foi aplicado as condições apropriadas de cultivo para isolar as bactérias capazes de usar a glicerina como substrato.

A glicerina mostrou ser um interessante, barato e abundante fonte de carbono para estimular bactérias redutoras de sulfato. A conversão microbiana de glicerol em processos de redução biológica de sulfato pode ser uma alternativa econômica e ambiental para biorremediação de áreas impactadas por metais pesados e para recuperação de metais, agregando valor a este subproduto e contribuindo comercialmente com as energias renováveis.