UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
MESTRADO EM BIOQUÍMICA

Influência da fonte de carbono na produção de biosurfactante por espécies de *Pseudomonas* isoladas de efluente agroindustrial

DANIELLE DOS SANTOS TAVARES PEREIRA

Orientadora: Dr[a] Maria da Paz Carvalho da Silva
Co-orientadora: Dr[a] Ana Maria Queijeiro López
Co-orientador: Dr Nicácio Henrique da Silva

RECIFE - 2007

DANIELLE DOS SANTOS TAVARES PEREIRA

Influência da fonte de carbono na produção de biosurfactante por espécies de *Pseudomonas* isoladas de efluente agroindustrial

Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de mestre em Bioquímica pela Universidade Federal de Pernambuco.

Aprovado por: Dr[a.] Maria Elisabeth Cavalcanti Chaves
Dr. José Luiz de Lima Filho
Dr[a.] Maria de Mascena Diniz Maia

Data: 27/02/2007

Pereira, Danielle dos Santos Tavares

Influência da fonte de carbono na produção de biosurfactante por espécies de Pseudomonas isoladas de efluente agroindustrial / Danielle dos Santos Tavares Pereira. – Recife : O Autor, 2007.

38 folhas: il., fig.

Dissertação (mestrado) – Bioquímica - Universidade Federal de Pernambuco. CCB, 2007.

Inclui bibliografia e anexos.

1. *Pseudomonas*. 2. Biosurfactante 3. Óleo de milho. I. Título

577.1 CDU (2.ed.) UFPE
572 CDD (22.ed.) CCB – 2007-083

Ata da defesa de dissertação da Mestranda **Daniele dos Santos Tavares Pereira,** realizada em 27 de fevereiro de 2007, como requisito final para obtenção do título de Mestre em Bioquímica.

Às 15:10 minutos do dia 27 de fevereiro de 2007, foi aberto, na Sala de Aulas do LIKA/UFPE, o ato de defesa de dissertação da mestranda **Daniele dos Santos Tavares Pereira,** aluna do Curso de Mestrado em Bioquímica/CCB/UFPE. Iniciando os trabalhos, a Profa. Dra. Maria da Paz Carvalho da Silva, na impossibilidade da presença da Coordenadora do Curso supra citado, Profa. Dra. Vera Lúcia de Menezes Lima, fez a apresentação da aluna, de sua orientadora, ela própria, de sua Co-Orientadora, Profa. Dra. Ana Maria Quyeijeiro Lopez, do Depto. de Biologia da UFAL, e Prof. Dr. Nicácio Henrique da Silva, do Depto. de Bioquímica/CCB/UFPE, e da Banca Examinadora composta pelos professores doutores: Maria da Paz Carvalho da Silva, na qualidade de Presidente, José Luiz de Lima Filho, Maria Elizabeth Cavalcante Chaves, os três do Depto. de Bioquímica, e Maria de Mascena Diniz Maia, do Depto. de Genética/UFRPE. Após as apresentações, a Sra. Presidente convidou a aluna para a apresentação de sua dissertação intitulada: **"Influência da fonte de carbono na produção de biosurfactantes por espécies de *Pseudomonas* isoladas de efluente agroindustrial"**, e informou que de acordo com o Regimento Interno do Curso, a candidata disporia de até 50 (cinqüenta) minutos para apresentação do trabalho e o tempo de argüição para cada examinador, juntamente com o tempo gasto pela aluna para responder às perguntas seria de 30 (trinta) minutos. A aluna procedeu a explanação e comentários acerca do tema em 35 (trinta e cinco) minutos. Após a apresentação da mestranda, a Sra. Presidente convidou a Banca Examinadora para ocupar seus lugares e passou a palavra ao primeiro examinador, a Profa. Dra. Maria de Mascena Diniz Maia, em seguida para a Profa. Dra. Maria Elizabeth Cavalcante Chaves, e finalmente para o Prof. Dr. José Luiz de Lima Filho, os quais agradecerem o convite, fizeram alguns comentários e sugestões. Ao final de suas respectivas argüições, os referidos professores deram-se por satisfeitos. Em seguida, a Sra. Presidente usou da palavra para tecer alguns comentários, agradecer à Banca Examinadora e parabenizar a candidata. Finalmente, a sessão foi suspensa para proceder ao julgamento pela Banca Examinadora, a qual se reuniu na Secretaria do LIKA/UFPE. Após alguns comentários, a Banca decidiu, por unanimidade, conceder a menção "**Aprovada com Distinção**". Nada mais havendo a tratar, lavrei a presente ata que vai assinada por mim, Secretário, e demais membros da Banca Examinadora. Recife, 27 de fevereiro de 2007.

EM TEMPO:
ONDE SE LÊ:
DANIELE DOS SANTOS TAVARES PEREIRA,
LEIA-SE: DANIELLE DOS SANTOS TAVARES PEREIRA.
Em: 27/02/2007

"À minha mãe, Maria José dos Santos Pereira, pelo apoio, compreensão, amizade, incentivo e amor".
Dedico

AGRADECIMENTOS

A Capes pelo auxílio financeiro concedido;

À Profa. Dra. Ana Maria Queijeiro López, Departamento de Química, Instituto de Química e Biotecnologia – Universidade Federal de Alagoas, por todos estes anos de amizade, orientações e concessões;

Ao Prof Dr Nicácio Henrique da Silva, Departamento de Bioquímica - UFPE, pela atenção e carinho;

À Profa. Dra. Vera Lucia de Menezes Lima, por sua dedicação na coordenação do curso de Mestrado em Bioquímica;

A todos os meus novos colegas de curso turma 2005, e em especial a Jayra Dantas, Chirleanny Mendes, Flávia Pereira, Paula Hirakawa, Érika Maria, Alessandra Mattos e Renata Fabiana;

Aos técnicos de Laboratório do Departamento de Bioquímica – UFPE, em especial a Sr. João Virgílio, pelo apoio e amizade;

Ao secretário do Departamento de Bioquímica – UFPE, José Miron, pela amizade e disposição em ajudar em todos os momentos;

A todos os meus amigos do Laboratório de Bioquímica do Parasitismo Vegetal e Microbiologia Ambiental, Instituto de Química e Biotecnologia – UFAL, em especial a Amanda Lys dos Santos Silva, pela amizade e ajuda na realização deste trabalho.

"Senhor, concedei-me a sabedoria para aceitar as coisas que não posso modificar, coragem para modificar aquelas que posso e discernimento para perceber a diferença".

São Francisco de Assis

LISTA DE FIGURAS

LISTA DE TABELAS

Capítulo 1. Influences of the carbon source in the growth and production of biosurfactant by Pseudomonas species isolated from agri-industrial effluent

ÍNDICE

RESUMO

Os biosurfactantes possuem diversas vantagens em relação aos surfactantes sintéticos, tais como: baixa toxicidade, biodegradabilidade, grande diversidade química e estrutural. Este estudo objetivou: 1) Isolar bactérias do gênero *Pseudomonas* de efluente agroindustrial; 2) Detectar a produção *in vitro* do biosurfactante ramnolipídico; 3) Verificar a utilização de óleo de milho na produção de biosurfactante em cultura líquida e 4) Caracterizar parcialmente o biosurfactante produzido sobre óleo de milho por cromatografia de camada delgada (CCD). Três bactérias do gênero *Pseudomonas* foram isoladas do efluente proveniente da lavagem de cana de açúcar da S.A. Usina Coruripe Açúcar e Álcool (Estado de Alagoas) e identificadas em nível de espécie, utilizando o kit API20E (BioMérieux, France), como sendo *P. aeruginosa, P. putida* e *P. fluorescens*. O meio de Siegmund & Wagner (SW) foi usado para caracterizar as linhagens produtoras de ramnolipídios sendo a produção deste biosurfactante *in vitro* evidenciada apenas para a espécie de *P. aeruginosa*. A produção de ramnolipídio em meio liquido pelo isolado de *P. aeruginosa*, tendo como fonte de carbono 1 % de óleo de milho, revelou uma maior concentração (200,58 mg/L) com 120 h de incubação, durante a fase estacionária, embora nenhuma alteração acentuada do pH tenha sido verificada. O biosurfactante foi também caracterizado por meio de técnicas de cromatografia de camada delgada, o qual foi possível ser detectado e cujos valores de R*f* foram semelhantes a aqueles descritos na literatura para as diferentes formas estruturais de ramnolipídios.

Palavras chaves: *Pseudomonas*, biosurfactante

ABSTRACT

The biosurfactantes possess many advantages in relation to the synthetic, surfactants, such as: low toxicity, biodegradability, great chemical and structural diversity. The objective of were: 1) To isolate bacteria of *Pseudomonas* genus in agri-industrial effluent; 2) To detect the production *in vitro* of the rhamnolipidic biosurfactant; 3) To verify the use of maize oil in the production of biosurfactant in liquid culture and 4) To characterize partially the biosurfactant produced on maize oil for thin layer chromatography (TLC). Three bacteria of the *Pseudomonas* genus were isolated effluent hamming from the laudering of sugar cane of the SA Coruripe Sugar and Alcohol (Alagoas/Brazil) and identified in species level, using kit API20E (BioMérieux, France), as being *P. aeruginosa*, *P. putida* and *P. fluorescens.* The Siegmund & Wagner medium was used to characterize the producing strains of rhamnolipid being the production of this biosurfactante *in vitro* evidenced only for the *P. aeruginosa* species. The production of rhamnolipid in liquid medium for the isolated *P. aeruginosa* using maize oil revealed the highest concentration (200.58 mg/L) with 120 h incubation, when the stationary phase revealed as carbon source clear, even so no alteration accented of pH has been verified. The biosurfactante also was characterized thin layer chromatography that was possible to be detected and the R*f* values of been similar to those described in literature for the different structural forms of rhamnolipid.

Kew words: *Pseudomonas, b*iosurfactante

1. INTRODUÇÃO

1.1. SURFACTANTE OU BIOSURFACTANTE

Surfactante é uma palavra derivada da expressão "surface active agent", termo que significa, literalmente, agente de atividade superficial. Em outras palavras, um surfactante é um composto caracterizado pela capacidade de alterar as propriedades superficiais e interfaciais de um líquido. Outra propriedade fundamental dos surfactantes é a tendência de formar agregados chamados micelas que, geralmente, formam-se a baixas concentrações em água (TIEHM, 1994; BERING *et al.,* 2004).

A maioria dos surfactantes produzidos atualmente é sintetizada a partir de derivados de petróleo e requerem tanto etapas complexas de síntese, quanto várias etapas de purificação (BODOUR & MILLER-MAIER, 1998). As moléculas de surfactantes sintético são usualmente classificadas de acordo com a natureza química do grupo polar em: catiônico, aniônico e tipos não aniônicos (PARRA *et al*., 1989).

De acordo com dados de SARNEY & VULFSON (1995), a produção mundial de surfactante excede três milhões de toneladas, o que representa em termos financeiros um valor estimado de US$ 4 bilhões (**Figura 1**). Atualmente, a indústria de detergentes é o maior consumidor deste produto. Contudo, os surfactantes biológicos ou biosurfactantes também podem ser utilizados em formulações de detergentes, visto que estes são compatíveis com a variedade de enzimas utilizadas como biodetergentes (ROSENBERG & RON, 1999). Na área bioquímica, os surfactantes são empregados na investigação das propriedades moleculares de proteínas e de lipoproteínas de membranas, agindo como agentes solubilizantes. Estes surfactantes químicos em sua grande maioria não são biodegradáveis, o que representa um sério problema ambiental (LEE, *et al.,* 1999).

Em contraste com seus análogos produzidos quimicamente, os biosurfactantes possuem vantagens especiais, como baixa toxicidade, biodegradabilidade, biodigestibilidade, produção a partir de vários substratos e grande diversidade química e estrutural. São mais efetivos do que os surfactantes convencionais, pois reduzem a tensão superficial e interfacial em menores concentrações. Apresentam tolerância às variações de temperatura, pH e força iônica, podendo ser utilizado em ambientes sob condições adversas (TURKOVISKAYA *et al.*, 2001; VANCE-HARROP *et al.*, 2003). Todavia, a maior vantagem que os biosurfactantes têm a oferecer em relação aos surfactantes químicos é sua aceitabilidade ecológica (HABA *et al*., 2000).

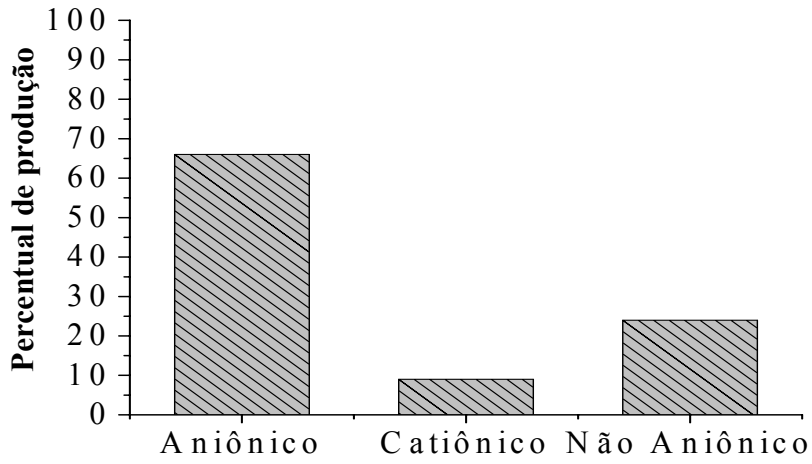

Figura 1. Tipos de surfactantes usados na indústria moderna (Fonte: SARNEY & VULFSON, 1995) Modificado.

O papel fisiológico dos biosurfactantes ainda não foi desvendado totalmente, porém algumas funções têm sido atribuídas a este grupo de moléculas, como: emulsificação e solubilização de hidrocarbonetos ou compostos insolúveis em água; transporte de hidrocarbonetos; aderência e liberação de células a superfícies e atividade antimicrobiana (ROSENBERGUE & RON, 1999).

Os biosurfactantes não são amplamente empregados em processos industriais devido ao alto custo de produção, associados aos métodos ineficientes de recuperação do produto e ao uso de substratos dispendiosos. No entanto, o problema econômico da produção de biosurfactantes pode ser significativamente reduzido através do uso de fontes alternativas de nutrientes facilmente disponíveis e de baixo custo (KIM *et al.*, 2000 citado por FARIAS & SARUBBO, 2004). Por exemplo, efluentes provenientes da agroindústria apresentam um elevado índice de carboidratos e lipídeos que podem ser utilizados por microrganismos na produção de biosurfactantes. Entretanto, são poucas as pesquisas acerca deste tipo de substrato como fonte de nutriente na produção de moléculas com propriedades tensoativas (3& PASTORE, 2004).

Os biosurfactantes são classificados de acordo com sua composição química e sua origem microbiana. As principais classes incluem glicolipídeos, lipopolissacarídeos, lipopeptídeos, fosfolipídeos, ácidos graxos e lipídeos neutros (BOGNOLO, 1999). A porção apolar dos biosurfactantes é freqüentemente uma cadeia hidrocarbonada saturada ou insaturada e/ou hidroxilada, como nos ácidos graxos ou álcoois graxos, respectivamente, enquanto a porção polar pode ser um mono, oligo ou polissacarídeo, peptídio ou proteína (LANG, 2002). Estas características tornam os biosurfactantes um produto biotecnológico adequado para uma ampla gama de aplicações industriais envolvendo: detergência, emulsificação, lubrificação, capacidade espumante,

capacidade molhante, solubilização e dispersão.

1.2. O GÊNERO *PSEUDOMONAS* E A PRODUÇÃO DE GLICOLIPÍDIOS

As espécies de *Pseudomonas* pertencem ao super-reino das eubactérias, Filo Proteobacteria, Classe Gammaproteobacteria, Ordem Pseudomonadales, Família Pseudomonadaceae. Em geral são Gram negativas, na forma de bastonetes de 0,5-0,8 μm x 1-3 μm, móveis por flagelos polares ou laterais, não formadoras de esporos, não-fermentantes de carboidratos e capazes de respirar anaerobicamente na presença de nitratos como aceptores de elétrons. Todas as espécies desse gênero geram piruvato a partir da via de Entner-Doudoroff. Não são fotossintetizantes, são oxidases positivas, geralmente catalase positivas, apresentam cerca de 58 - 68 % de G - C em seu DNA, degradam arginina a ornitina e podem produzir pigmentos fluorescentes ou não, não requerendo fatores de crescimento. São capazes de crescer em uma variedade de nutrientes em ambientes dos mais variados tipos, sendo parasitas de plantas e animais, mutualistas ou saprófitas (HOLT *et al.*, 1999).

As espécies que produzem pigmentos fluorescentes são de muita importância em diversas áreas como fitopatologia, tecnologia de alimentos e controle biológico. *P. aeruginosa, P. fluorescens e P. putida* produzem pigmentos fluorescentes. No entanto, *P. aeruginosa* é uma bactéria oportunista facilmente identificável, desde que seu grupo é homogêneo do ponto de vista de critérios genéticos, tanto que seu genoma já foi caracterizado. *P.fluorescens*, contudo, é um grupo taxonômico bastante heterogêneo, com muitas subespécies referidas como biótipos ou biovariedades, e por isso em geral algumas são referidas como *Pseudomonas* sp. O mesmo pode ocorrer com *P. putida.* No entanto, enquanto a primeira é capaz de crescer a 41 ºC, reduzir nitratos e produzir piocianina (azul na luz visível) e fluoresceína (pioverdina), o que intensifica sua fluorescência a 366 nm, as duas últimas não são capazes de crescer naquela temperatura, raramente são patogênicas ou reduzem nitratos, e nem produzem piocianina. A fluoresceína absorve luz a 254 nm e atua como sideróforo, portanto, a ausência de ferro intensifica sua fluorescência. Outros pigmentos como piorubrina (vermelho) e piomelanina (marrom) podem ser produzidos, mas não produzem fluorescência (HOLT *et al.*, 1999; BOSSIS *et al.*, 2000).

A produção de biosurfactante não está relacionada apenas com a assimilação de substratos insolúveis, pois se sabe que a produção de glicolipídios ocorre tanto sobre substratos solúveis como insolúveis. A síntese de tensoativos pela célula microbiana parece obedecer mais a uma necessidade fisiológica do organismo, como por exemplo, a adesão e hidrofobicidade da membrana celular de bactérias, em condições limitadas de nutrientes (ARINO *et al.,* 1996; BONILLA *et al.*, 2005).

Glicolipídios emulsificantes são cadeias de ácidos graxos ou hidroxiácidos graxos ligadas a uma ou mais moléculas de carboidratos como glicose, manose, trealose, ramnose. Estes bioemulsificadores são moléculas de baixo peso molecular capazes de aumentar a solubilidade aparente dos hidrocarbonetos organizados nas cavidades de micelas hidrofóbicas (KARANTH *et al.,* 1999). A produção de biosurfactante depende da natureza do substrato, das condições de pH, temperatura, agitação, da concentração de íons como, Mg, P, Fe, K e da atividade da biomassa (DESAI & BANAT, 1997).

Os ramnolipídios, biosurfactantes aniônicos produzidos por diferentes espécies de *Pseudomonas,* representam um dos glicolipídios mais estudados devido à sua aplicabilidade em diversas áreas, como por exemplo, na biorremediação de derivados de petróleo (LANG & WULLBRANDT, 1999; WARD *et al.*, 2003). Essa é, atualmente, uma das áreas onde surfactantes de origem microbiana são mais utilizados (ONWURACH, 2004).

BURGER *et al.* (1963), verificaram que a síntese enzimática de ramnolipídios produzidos por *P. aeruginosa* ATCC7700 é decorrente da reação seqüencial de glicosil transferases específicas (ramnosil transferase 1 e rammnosil transferase 2) e que a timidina-difosfato-L-ramnose (TDP-L-ramnose) é um eficiente ramnosil doador envolvido na síntese deste glicolipídio. A síntese do monoramnolipidio (R1) é catalisada pela ramnosil transferase 1 (rhlAB) onde ocorre a transferência da ramnose ativada (dTDP-L-ramnose) para o acido graxo aceptor β-hidroxidecanoil-β-hidroxidecanoato. Posteriormente, pela ação da ramnosil transferase 2 (rhlC) ocorre a transferência da ramnose ativada para a molécula de monoramnolipidio (**Figura 2**).

A síntese de ramnolipídios em *P. aeruginosa* tem sido correlacionada com o acumulo intracelular do ácido polihidroxialcanoico (PHAs). Isto pode ser possível devido ao fato de tanto a porção lipídica da molécula de ramnolípidios quanto a síntese de PHAs ocorrerem de forma geral pela via de síntese de ácidos graxos, a nível da enzima β-cetoacil redutase (RhlG), a partir da redução do intermediário cetoacilo-ACP (LANG, 2002).

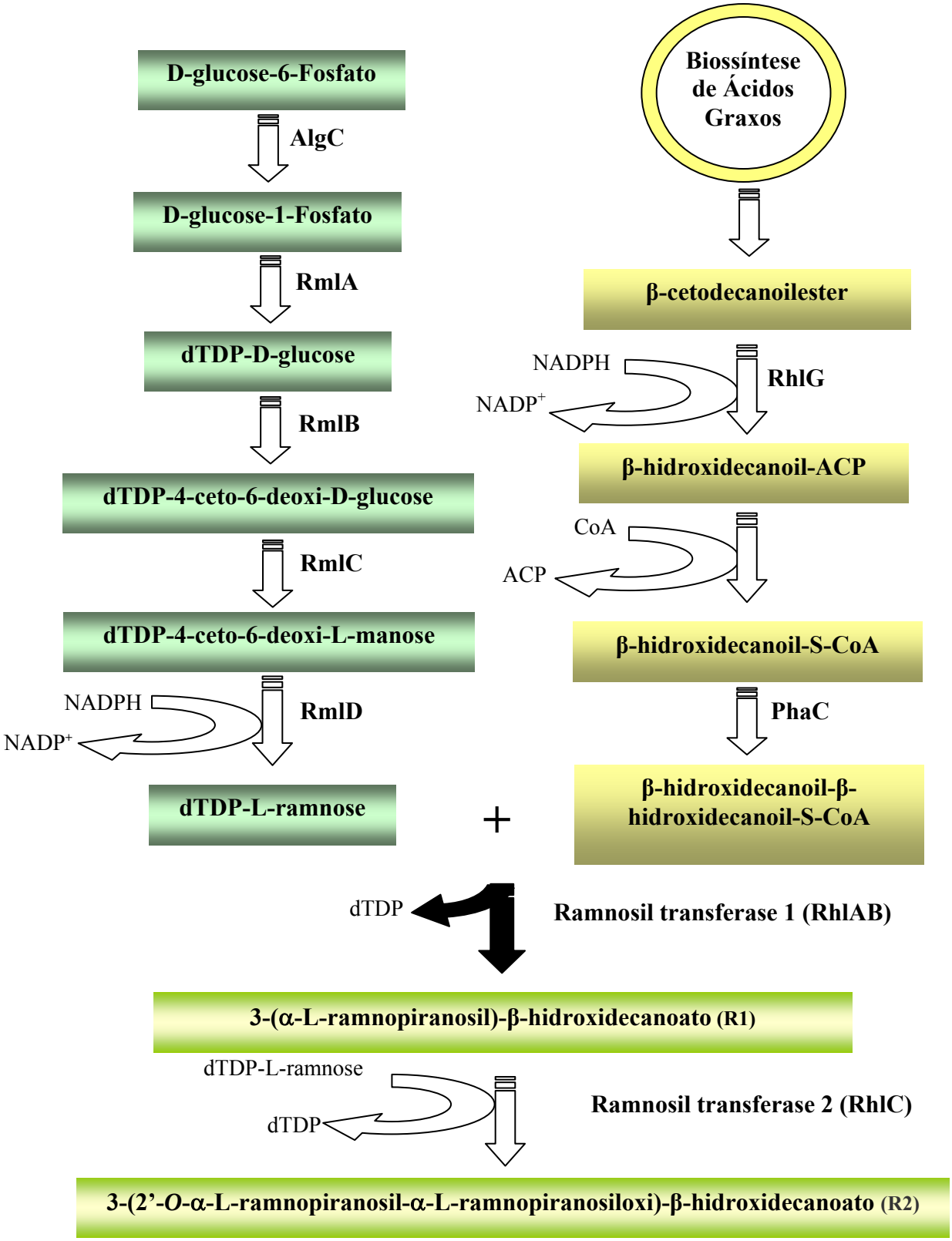

Figura 2. Biossíntese de ramnolipídios relacionada à síntese de ácidos graxos (Fonte: MAIER & SÓBERON-CHAVÉZ, 2000).

A estrutura do ramnolipídio varia de acordo com a espécie de *Pseudomonas* spp e o tipo de substrato. Os ramnolipídio do tipo R1 → [3-(α-L-ramnopiranosil)-β-hidroxidecanoato], e o ramnolipídio R2 → [3-(2'-*O*-α-L-ramnopiranosil-α-L-ramnopiranosiloxi)-β-hidroxidecanoato], são os principais glicolipídios produzidos em culturas líquidas. Enquanto que a formação do ramnolipídio R3 → 3-[3'-(α-L-ramnopiranosil-hidroxidecanoil)-β-hidroxidecanoato], e o ramnolipídio R4 → 3-[3'-(2''-*O*-α-L-ramnopiranosil-α-L-ramnopiranosil-hidroxidecanoil)-β-hidroxidecanoato], (**Figura 3**), contendo uma molécula do ácido ß-hidroxidecanóico com uma ou duas unidades de ramnose, respectivamente, são produzidos exclusivamente por algumas linhagens restritas (SCHENK *et al*., 1995; KOCH *et al*., 1991).

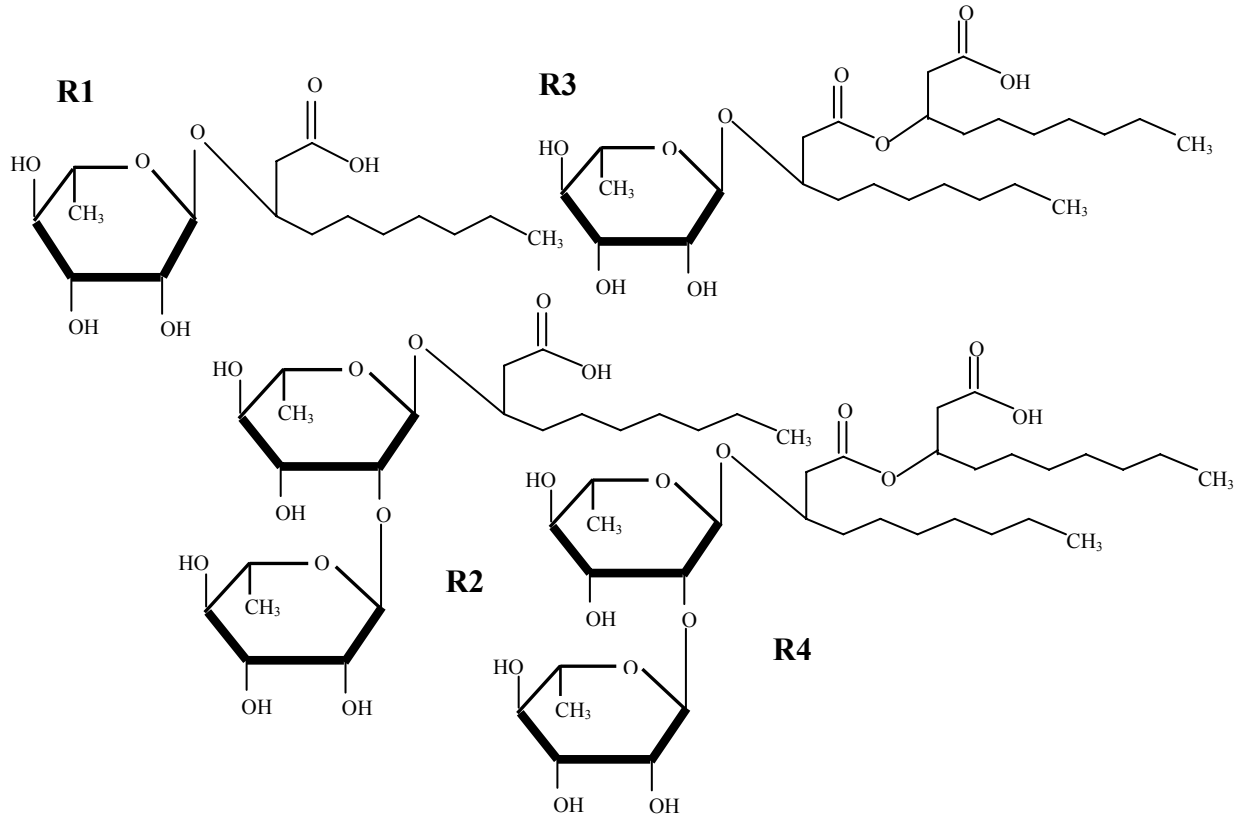

Figura 3. Diferentes tipos de ramnolipídeos produzidos por *Pseudomonas aeruginosa* (SCHENK *et al*., 1995; KOCH *et al*., 1991).

O complexo ramnosil transferase 1 é constituído pela proteína RhlA (32,5 kDa) localizada na membrana plasmática e pela proteína RhlB (47 kDa) localizada na região periplasmática (DESAI & BANAT, 1997). Os genes *rhlA* e *rhlB* envolvidos na síntese de ramnolipídios por *P. aeruginosa* são regulados por dois sistemas hierárquicos de sensor de quorum *rhl* e *las* (SULLIVAN, 1998) (**Figura 4**). A expressão do gene *rhlC* codifica a síntese da ramnosil transferase 2, proteína de massa molecular igual 35,9

kDa constituída por 35 aminoácidos. Este gene é regulado ordenadamente pelo gene *rhlAB* e pelo sensor de quorum *rhl* (LANG, 2002).

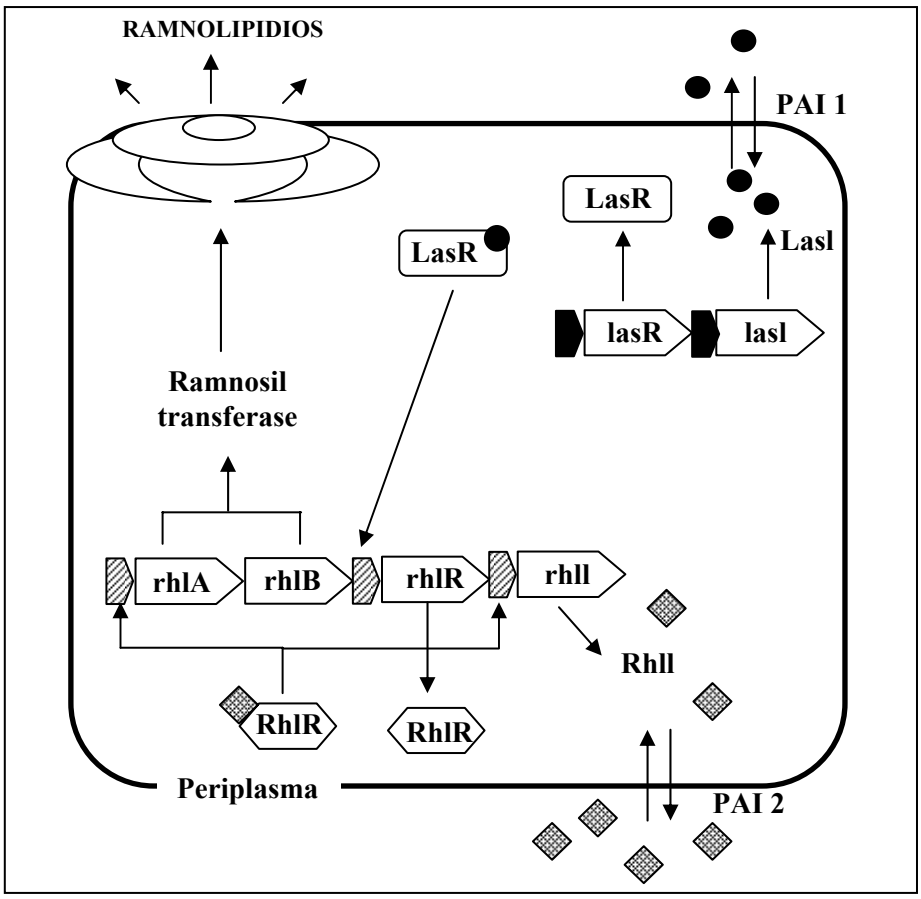

Figura 4. Modelo esquemático dos genes envolvidos na síntese de ramnolipideos por *P. aeruginosa.* Promotor auto indutor: PAI 1 ou *N*-(3-oxododecanoil) homoserina lactona, e PAI 2 ou *N*-butiril homoserina lactona (OCHSNER & REISER, 1995; SULLIVAN,1998).

O sistema *las* consiste de um regulador de transcrição, LasR, e sua molécula sinalizadora, *N*-(3-oxododecanoil) homoserina lactona, codificada pelo gene *lasI.* O sistema *rhl* consiste da proteína RhlR e RhlI sintase, que está envolvida na produção de *N*-butiril homoserina lactona (SULLIVAN,1998; LANG &. WULLBRANDT, 1999). Esta por sua vez interage com RhlR para ativar a expressão de *rhl*AB, o operón que codifica a enzima ramnosil transferase necessária para a produção de ramnolipídios (OCHSNER & REISER, 1995; DÉZIEL *et al.*, 2003). A presença destes componentes reduz a tensão superficial que permite às células de *P. aeruginosa* unir-se e desunir-se a diferentes substratos (KÖHLER *et al*., 2000).

De acordo com BRINT & OHMAN (1995) e PESSI & HAAS (2000), o sistema RhlRI é necessário para indução da expressão de fatores como: protease alcalina,

piocianina, cianeto de hidrogênio, lectina e elastase. Já o gene LasR está envolvido com a síntese de biofilme ou biopelícula. É enorme a diversidade de espécies microbianas dentre bactérias, fungos, vírus e microalgas, que podem estar presentes nos biofilmes, quer nos habitats naturais, quer nos criados artificialmente (BALIELLAS, 2004). SCHOOLING *et al.* (2004) verificaram que a adição de ramnolipídio produzido por *P. aeruginosa* mutante PAO inibiu a formação de biofilme, impedindo assim a dispersão microbiana.

TULEVA *et al.* (2002) relataram a produção de biosurfactante por uma estirpe de *P. putida* 21BN cultivada sobre substratos pouco solúveis, como o hexadecano, ou solúveis, como glicose, cuja natureza química do surfactante é ramnolipídica. HEALY *et al.* (1996), verificaram a produção de ramnolipídio, contendo grupos éster e carboxil ligados a porção lipídica hidrofóbica, por *P. fluorescens* (NCIMB117/2) cultivada sobre óleo de oliva virgem. NIELSEN *et al.* (1999) identificaram um novo dipeptídio cíclico com propriedades surfactante e antifúngica, produzido por *P. fluorescens* DR54 denominado viscosamida que apresenta propriedades distintas em relação ao biosurfactante viscosina produzido por algumas linhagens desta espécie.

As aplicações biotecnológicas dos ramnolipidios são inúmeras, principalmente para aplicação em biorremediação em casos de contaminação envolvendo hidrocarbonetos policíclicos aromáticos (HPA), hidrocarbonetos bifenóis policlorados (HBP) e de petróleo, sendo o petróleo em estado bruto considerado o maior poluente marinho, e os seus subprodutos, como gasolina, óleo diesel e óleos lubrificantes, os mais freqüentes poluentes orgânicos de solos e aqüíferos subterrâneos (NAITALI *et al*., 1999).

1.3. BIORREMEDIAÇÃO DE DERIVADOS DE PETRÓLEO

Biorremediação é a habilidade de organismos vivos em transformar ou mineralizar contaminantes orgânicos gerando substâncias menos nocivas, que possam ser integradas ao ciclo biogeoquímico natural. Contudo, a biodegradabilidade destes contaminantes é influenciada por fatores como: oxigênio, pH, presença de macro e micro nutrientes, características físico-químicas do histórico da poluição do contaminante ambiental e das partículas de solo ou outras às quais os organismos e contaminantes possam estar adsorvidos (MARGESIN & SCHINNER, 2001).

A biorremediação apresenta muitas vantagens por ser um tratamento natural e ambientalmente seguro e possuir riscos mínimos de exposição aos poluentes, além de ser uma alternativa barata. Porém, há algumas desvantagens que devem ser levadas em consideração, como, por exemplo, a transformação incompleta dos contaminantes e a

introdução de organismos exógenos, sendo as conseqüências desse fato ainda uma incógnita para os pesquisadores (MABIC, 2005).

A maioria das substâncias contaminantes apresenta diferentes grupos funcionais tais como: OH, Cl, NH_2, NO_2 e SO_3. Estes por sua vez comportam-se como doadores de elétrons sendo oxidados ou em alguns casos mineralizados por diferentes espécies microbianas. Alguns dos metabólitos intermediários produzidos nestas reações são assimilados como fonte de carbono para o crescimento microbiano (WILSON & BOUWER, 1997).

A causa fundamental da difícil degradação dos compostos hidrofóbicos de alto peso molecular é sua baixa solubilidade em água, aumentando assim a sua adsorção ao solo de forma irreversível, limitando a disponibilidade destas substâncias ao ataque de microrganismos (KNAEBEL *et al.,* 1994). Neste contexto, a utilização de um agente emulsificante aumenta os índices de biodegradação, visto que os problemas relativos á baixa solubilidade em água, a alta fixação e a baixa transferência desses poluentes da fase sólida para a fase aquosa são minimizados.

KOCH *et al.* (1991), descreveram que o aumento da biodegradação de alcanos pela adição de ramnolipídios deve-se possivelmente a diminuição da hidrofobicidade da superfície celular, após a extração do lipopolissacarídio do envelope celular pelo ramnolipídio, possibilitando um contato direto entre as células e o hidrocarboneto. Os ramnolipídios do tipo R1 e R2 estão relacionados com o transporte e assimilação de hidrocarbonetos por *P. aeruginosa* (OZDEMIR & MALAYOGLU, 2004).

KUIPER *et al.* (2004), isolaram a espécie *Pseudomonas putida* a partir de raízes de plantas que cresciam em um solo contaminado com hidrocarbonetos policíclicos aromáticos. O isolado demonstrou atividade biosurfactante ao fim da fase exponencial. As análises cromatográficas do sobrenadante da cultura mostraram dois picos com atividade redutora da tensão superficial, demonstrando a produção de dois tipos de biosurfactantes. Apesar da grande maioria dos estudos destinados ao isolamento e identificação de microrganismos serem em locais contaminados, bactérias produtoras de biosurfactante também são encontradas em ambientes sem registro de contaminação prévia. JENNINGS & TANNER (2000), observaram que até 35 % da população microbiana heterotrófica de um solo não contaminado era produtora de tensoativos.

PROVIDENTI *et al.* (1995), avaliaram os efeitos da adição de biosurfactante produzido por *P. aeruginosa* UG2 na mineralização do fenantreno, onde foram conduzidos três experimentos: 1º mineralização de fenantreno por microrganismos com a adição do biosurfactante; 2º mineralização do fenantreno utilizando apenas o biosurfactante e 3º acompanhamento da mineralização do fenantreno por outra espécie do gênero *Pseudomonas* com adição do biosurfactante. Os resultados deste trabalho mostraram que: os microrganismos degradadores de fenantreno não são exclusivos de

solos contaminados com este composto; ao final do experimento não houve variação significativa entre a porcentagem de fenantreno degradada nos solos inoculados e nos não inoculados, porém, o início da degradação de fenantreno foi mais rápido nos solos inoculados.

ZHANG & MILLER (1992), investigaram o potencial de biodegradação de octadecano por *P. aeruginosa* ATCC9027 com adição de ramnolipídios. A mensuração da tensão superficial revelou que *P. aeruginosa* não produz ramnolipídio durante o crescimento sob octadecano em meio de sais minerais, mineralizando apenas 5 % da concentração do substrato inicial de 1,5 mg/L. A adição de ramnolipídios ao meio mineral aumentou a mineralização de octadecano para 10,2, 12,8 e 19,9 %, nas concentrações de 100 mg/L, 125 mg/L e 300 mg/L, respectivamente.

SANDRIN *et al.* (2000), verificaram o efeito de ramnolipídios no crescimento de *Burkholderia* sp sob naftaleno na presença de Cd^{2+}. O crescimento de *Burkholderia* sp não é alterado pela adição de ramnolipídio, sendo inibido na presença de 89 μM de Cd^{2+}. A eliminação da toxicidade do Cd^{2+} foi observada pela adição de 890 μM de ramnolipidio, favorecendo assim o crescimento de *Burkholderia* sp na presença de Cd^{2+}. Estes resultados sugerem a utilização de ramnolipídios em consórcios para biorremediar áreas que contenham diferentes contaminantes. Isto se deve a afinidades deste biosurfactante por cátions como $K^{+} < Mg^{2+} < Mn^{+2} < Ni^{2+} < Co^{2+} < Ca^{2+} < Hg^{2+} < Fe^{+3} < Zn^{2+} < Cd^{2+} < Pb^{2+} < Cu^{2+} < Al^{3+}$ (MULLIGAN, 2005).

SHIN & KIM (2004), estudaram o efeito de surfactante químico e biológico na remoção de fenantreno e diesel em coluna de solo experimental. O biosurfactante ramnolipídio foi capaz de remover 67 % de fenantreno e 60 % de óleo diesel do solo. Em contraste, com o surfactante não iônico Tween 80, que apresentou baixa remoção destes contaminantes.

URUM *et al.* (2004), verificaram a remoção de óleo bruto de diferentes frações de partículas de solos tratados com ramnolipídios e dodecil sulfato de sódio (SDS). Tanto o ramnolipídio quanto o SDS apresentaram remoção de 6 % de óleo bruto, com uma concentração micelar crítica de 0.02 e 0.2 % massa, respectivamente.

Surfactantes químicos como o Triton X100 e o Tween 80, têm apresentado bons resultados em processos de biorremediação. Por outro lado, a hidrólise do SDS gera íons HSO_4^- que conduzem a acidificação do meio, afetando negativamente a população microbiana (RON & RONSENBERG 2002; TIEHM *et al.*, 1994). Desta forma, a utilização de tensoativos no tratamento de áreas contaminadas requer a identificação de organismos capazes de degradar tanto o contaminante quanto o surfactante. Segundo RON & ROSENBERG (2001) os microrganismos degradadores de contaminantes hidrofóbicos são capazes de crescer na presença de biosurfactantes produzidos por outras espécies, facilitando assim sua bioassimilação e biodegradação.

A utilização de tensoativos no tratamento de contaminantes como: hidrocarbonetos policíclicos aromáticos e hidrocarbonetos bifenóis policlorados, têm apresentado bons resultados em estudos laboratoriais e em campo. Estes contaminantes são substâncias que apresentam propriedades cancerígenas e mutagênicas, cuja incorporação na cadeia alimentar pode provocar danos irreversíveis, ao mesmo tempo em que representam à fração de petróleo que é mais recalcitrante aos métodos convencionais de remediação (KIYOHARA *et al.,* 1982; MEZIE *et al.*, 1992).

2. JUSTIFICATIVA

As bactérias pertencentes ao gênero *Pseudomonas* são reconhecidas por sua habilidade em degradar compostos de difícil assimilação por outros organismos, incluindo desde moléculas simples a compostos orgânicos complexos (HASANUZZAMAN *et al.,* 2004). Espécies deste gênero apresentam diversas aplicações biotecnológica, em especial na área ambiental, pois são capazes de sintetizar uma variedade de enzimas sob as mais diversas condições de cultivo (WARD *et al.,* 2003).

Ramnolipídios produzido por *Pseudomonas spp* é um dos biosurfactantes glicolipídico mais estudado devido a sua aplicabilidade em biorremediação em casos de contaminação envolvendo petróleo e os seus subprodutos como: gasolina, óleo diesel e óleos combustíveis (BANAT, 1994). Entre as tecnologias utilizadas neste tipo de biorremediação podemos citar: a utilização de surfactantes químicos e biológicos. Contudo quando utilizado surfactante químico é interessante identificar organismos capazes de degradar tanto o contaminante quanto o surfactante para que não haja acúmulo de outro composto xenobiótico.

O estudo de biosurfactantes é de grande relevância tanto industrial quanto para a comunidade científica na área de microbiologia, bioquímica, biotecnologia e biorremediação. Além disso, este estudo justifica-se pelo aumento do interesse industrial no desenvolvimento de tecnologias que não causem danos ao ambiente e que sigam a novas legislações ambientais, visando assim fontes alternativas de obtenção de produtos biológicos em substituição aos produtos existentes.

3. REFERENCIAS BIBLIOGRÁFICAS

ARINO, S., MARCHAL, R., VANDECASTEELE, J.P. 1996. Identification and production of rhamnolipdic biosurfactant by a *Pseudomonas* species. Appl. Microbial. Biotechnol., 45:162-168.

BALIELLAS, E.V. 2004. Estructura i junció dels biofilms fluvials: Implicacions en la dinamica del doc al riu i la producció de metabolits secundaris. Tese Doutorado: Faculdade de Biologia, Departamento de Ecologia, Universidade de Barcelona.

BANAT, I.M. 1994. Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation: a review. Bioresour. Tecnol., 5:1-12.

BEHRING, J.L., LUCAS, M., MACHADO, C., BARCELLOS, I.O. 2004. Adaptação no método do peso da gota para determinação da tensão superficial: Um método simplificado para a quantificação da CMC de surfactantes no ensino da química. Quím. Nova, 27:492-495.

BODOUR, A.A., MILLER-MAIER, R.M. 1998. Application of a modified drop-collapse technique for surfactant quantification and screening of biosurfactant-producing microorganisms. J. Microbiolog. Method., 32:273-280.

BOGNOLO, G. 1999. Biosurfactants as emulsifier agents for hydrocarbons. Colloids and Surfaces A: Physicochem. and Engineer. Aspects, 152:41-52.

BONILLA, M., OLIVARO, C., CORONA, M., VAZQUEZ, A., SOUBES, M. 2005. Production and characterization of a new bioemulsifier from *Pseudomonas putida* ML2. J. Appl. Microbiol., 98:456-463.

BOSSIS E., LEMANCEAU P., LATOUR X., GARDAN L. 2000. The taxonomy of *Pseudomonas fluorescens* and *Pseudomonas putida*: current status and need for revision. Agronomie, 20:51-63.

BRINT, J.M., OHMAN, D.E. 1995. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. J. Bacteriol., 177:7155-7163.

BURGER, M.M., GLASER, L., BURTON, R.M. 1963. The enzymatic synthesis of a rhamnose-contating glycolipid by extracts of *Pseudomonas aeruginosa*. T.J. Biolog. Chemist., 238:2595-2602.

DESAI, J.D., BANAT, I.M. 1997. Microbial production of surfactants and theircommercial potential. Microbiol. Molecl. Biol. Rev., 61:47–64.

DÉZIEL, E., LÉPINE, F., MILOT, S., VILLEMUR, R. 2003. rhlA is required for the producrion of a novel biosurfactante promoting swarming motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology, 149:2005-2013.

FARIAS, C.B.B., SARUBBO, L.A. 2004. Estudo das propriedades do biosurfactante produzido por *Candida lipolytica* cultivada em óleo de canola e glicose como substratos. In: Anais da VII[a.] Reunião Regional da SBBq e 2[nd] International Symposium in Biochemistry of Macromolecules and Biotechnology – SBBq, pp. 117-119.

HABA, E., ESPUNY, M.J., BUSQUETS, M., MANRESA, A. 2000. Screening and production of rhamnolipids by *Pseudomonas aeruginosa* 47T2 NCIB 40044 from waste frying oils. J. Appl. Microbiol., 88:379–387.

HASANUZZAMAN, M.; UMADHAY-BRIONES, K.M.; ZSIROS, S.; MORITA, N.; NODASAKA, Y, YUMOTO, I., OKUYAMA, H. 2004. Isolation, identification, and characterization of a novel, oil-degrading bacterium, *Pseudomonas aeruginosa* T1. Current Microbiol., 49:108-114.

HOLT, J.G., N.R. KRIEG, P.H.A. SNEATH, J.T. STALEY, S.T. WILLIAMS. Bergey's manual of determinative bacteriology. 9[a] edition. Williams and Wilkins, (ISBN 0-683-00603-7), 1999.

JENNINGS, E.M; TANNER, R.S. 2000. Biosurfactant-producing bacteria found in contaminated and uncontaminated Soils. Procedings of the 2000 Conference on Hazardous Waste Research. 299-306.

KANAEBEL, D.B., FEDERLE, T.W., McAVOY, D.C., VESTAL, J.R. 1994. Effect of mineral and organic soil constituents on microbial mineralization of organic compounds in a natural soil. Appl. Environm. Microbiol., 60:4500-4508.

KARANTH, N.G.R., DEO, P.G., VEENANADING, N.K. 1999. Microbial production of biosurfactants and their importance. Current Science On Line, 77:116-126.

KIYOHARA, H., NAGAO, K., YANA, K. 1982. Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. Appl. Environm. Microbiol., 43:454-457.

KOCH, A. K., KAPPELI, O., FIECHTER, A., REISER, J. 1991. Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. J. Bacterial., 173:4212-4219.

KÖHLER, T., CURTY, L.K., BARJA, F., VAN DELDEN, C., PECHERE, J.C. 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J. Bacteriol., 182:5990-5996.

KUIPER, I., LAGENDIJK, E.L., PICKFORD, R. DERRICK, J.D., LAMERS, G.E.M., THOMAS-OATES, J.E., LUGTENBERG, B.J.J., BLOEMBERG, G.V. 2004. Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilm. Mol. Microbiol., 51:97-113.

HEALY, M.G., DEVINE, C.M., MURPHY, R. 1996. Microbial production of biosurfactants. Resources Conservation and Recycling, 18:41-57.

LANG, S. 2002. Biological amphiphiles (Microbial biosurfactants). Current Opinion in Colloids & Interface Science, 7:12-20.

LANG, S., WULLBRANDT, D. 1999. Rhamnose lipids-biosynthesis, microbial production and applied. Microbiol. Biotecnology, 51:22-32.

LEE, Y., LEE, S.Y., YANG, J-W. 1999. Production of rhamnolipid biosurfactant by fed-batch culture of *Pseudomonas aeruginosa* using glucose as a sole carbon source. Biosci. Biotechnol. Biochem., 63:946-947.

MABIC: Malaysian Biotechnology Information Centre. 2005. Bioremediation: Nature's Way to A Cleaner Environment. 9. Disponível *on-line* em: www.bic.org.my. Acesso em: 15/02/06.

MAIER, R. M., SOBERÓN-CHÁVEZ, G. 2000. *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. Appl. Microbial., 54:625-633.

MARGESIN, R., SHINNER. F. 2001. Biodegradation and bioremediation of hidrocarbon in extreme environments. Appl. Microbiol. Biotechnol., 56:650-663.

MEZIE, C.A., POTOCKI, B.B., SANTODONATO, J. 1992. Exposure to carcinogenic PAHs in the environmenment. Environ. Sci. Technol., 26:1278-1284.

MULLIGAN, C.N. 2005. Environmental applications for biosurfactants. Environm. Pollution, 33:183-198.

NAITALI, M.B., RAKATOZAFY, H., MARCHAL, R., LEVEAU, J.Y., VANDECASTEELE, J.P. 1999. Diversity of bacterial strains degrading hexadecane in relation to the mode of substrate uptake. J. Appl. Microbiol., 86:421-428.

NIELSEN, T.H., CHRISTOPHERSEN, C., ANTHONI, V., SORENSE, J. 1999. Viscosamide, a new cyclic depsipeptide with surfactant and antifungal properties produced by *Pseudomonas fluorescens* DR54. J. Appl. Microbiol., 86, 80-90.

NITSCHKE, M., PASTORE, G.M. 2003. Avaliação de resíduos agroindustriais como substrato para a produção de biosurfactantes por *Bacillus*. Revista Biotecnol. Ciência e Desenvolvimento, 31:63-67.

OCHSNER, U.A., REISER, J. 1995. Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. Proc. Natl. Sci. USA, 92:6424-6428.

ONWURUAH, I.N.E. 2004. Modelling Cometabolism of petroleum hydrocarbon pollutants in soil by *Azotobacter vinelondu* in the obligate presence of *Pseudomonas* sp. Environm. Contamination Toxicol., 73:690-697.

OZDEMIR, G., MALAYOGLU, U. 2004. Wetting characteristics of aqueous rhamnolipids solutions. Colloids and Surfaces B: Biointerfaces, 39:1-7.

PARRA, J.L., GUINEA, J., MANRESA, M.A., ROBERT, M., MERCADÉ, M.E., COMELLES, F., BOSCH, M.P. 1989. Chemical characterization and physicochemical behavior of biosurfactants. JAOCS, 66:141-145.

PESSI, G., HAAS, D. 2000. Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum sensing regulators LasR and RhlR in *Pseudomonas aeruginosa,* J. Bacteriol., 182:6940-6949.

PROVIDENTI, M.A., FLEMNING, C.A., LEE, H., TREVORS, J.T. 1995. Effect of addition of rhamnolipid biosurfactants or rhamnolipid-producing *Pseudomonas aeruginosa* on phenanthrene mineralization in soil slurries. FEMS Microbiol. Ecol., 17:15-26.

RON, E.Z., ROSENBERG, E. 2001. Natural roles of biosurfactants. Enviromn. Microbiol., 3:229-236.

RON, E.Z., RONSENBERG, E. 2002. Biosurfactants and oil bioremediation. Current Opinion in Biotechnol., 13:249-252.

ROSENBERG, E., RON, E.Z. 1999 High- and low-molecular mass microbial surfactants. Appl. Microbiol. Biotechonol., 52:154-162.

SANDRIN, T.R., CHECH, A.M., MAIER, R.M. 2000. A rhamnolipid biosurfactant reduces cadmium toxicity during naphthalene biodegradation. Appl. Environm. Microbiol., 66:4585-4588.

SARNEY, D.B., VULFSON, E.N. 1995. Application of enzymes to the synthesis of surfactants. TIBTECH, 13:164-172.

SCHENK, T., SCHUPHAN, I., SCHMIDT, B. 1995. Higth performance liquid chromatographic determination of the rhamnolipids produced by *Pseudomonas aeruginosa*. J. Chromatog. A, 687:7-13.

SCHOOLING, S.R., CHARAF, U.K., ALLISON, D.G., GILBERT, P. 2004. A role for rhamnolipid in biofilm dispersion. Biofilms, 1:91-99.

SHIN, K., KIM, K. 2004. A biosurfactant-enhanced soil flushing for the removal of phenantrene and diesel in sand. Environm. Geochemist. and Health, 26:5-11.

SULLIVAN, E.R. 1998. Molecular genetics of biosurfactante production. Current Opinion in Biotechnol., 9:263-269.

THIEM, A. 1994. Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. Appl. Environm. Microbiol., 60:258-263.

TULEVA, B.K., IVANOV, G.R., CHRISTOVA, N.E. Biosurfactant production by a new *Pseudomonas putida* Strain. Z. Naturforsch, v. 57c, pp. 356-360, 2002.

TURKOVSKAYA, O.V., DMITRICIA, T.V., MURATOVA, A.YU. 2001. A biosurfactant producing *Pseudomonas aeroginosa* strain. Appl. Biochemist. and Microbiol., 37:71-75.

URUM, K., PEKDEMIR, T., ÇOPUR, M. 2004. Surfactants treatment of crude oil contaminated soils. J Colloid and Interface Sci., 276:456-464.

VANCE-HARROP, M.H., GUSMÃO, N.B., TAKAKI, G.M.C. 2003. New bioemulsifiers produced by *Candida lipolytica* using D-glucose and babassu oil as carbon sources. Brazilian J. Microbiol., 34:120-123.

WARD, O., SINGER, A., HAMME, J.V. 2003. Accelerated biodegradation of petroleum hydrocarbon waste. J. Indust. Microbiol. Biotechnol., 30:260-270.

WILSON, L.P., BOUWER, A.V. 1997. Biodegradation of aromatic compounds under mixed oxygen/denitrifying conditions: a review. J. Indust. Microbiol. Biotechnol., 18:116-130.

ZHANG, Y., MILLER, R.M. 1992. Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). Appl. Environm. Microbiol., 58:3276-3282.

4. OBJETIVOS

4.1. GERAL

Avaliar a produção de biosurfactante por diferentes isolados de *Pseudomonas* utilizando fontes alternativas de carbono.

4.2. ESPECÍFICOS

1. Isolar bactérias do gênero *Pseudomonas* de efluente agroindustrial;

2. Detectar a produção de biosurfactante ramnolipídico pelos isolados obtidos;

3. Verificar a utilização de óleos vegetais na produção de biosurfactante;

4. Caracterizar parcialmente o biosurfactante produzido sobre óleo de milho por cromatografia de camada delgada (CCD).

5. CAPÍTULO 1

Influences of the carbon source in the growth and production of biosurfactant by *Pseudomonas* species isolated from agri-industrial effluent

Artigo a ser submetido para publicação no periódico:
"FEMS Microbiology Letters"

Influences of the carbon source in the growth and production of biosurfactant by *Pseudomonas* species isolated from agri-industrial effluent

Danielle dos Santos Tavares Pereira [b,c], Amanda Lys Santos Silva[c], Nicácio Henrique da Silva[b], Ana Maria Queijeiro López[c,]*, Maria da Paz Carvalho da Silva[a,b]

[a]Setor de Bioquímica – LIKA, Universidade Federal de Pernambuco, Recife-PE, Brasil.
[b]Departamento de Bioquímica, Universidade Federal de Pernambuco, Recife-PE, Brasil.
[c]Departamento de Química, Instituto de Química e Biotecnologia, Universidade Federal de Alagoas, Maceió-AL, Brasil. BR 104 N, Km 97, Campus A.C. Simões, CEP 57072-970

Abstract

Three bacterial gram negative to present the growth in Siegmund and Wagner medium, were identify with *P. aeruginosa*, *P. fluorescens* and *P. putida*. The utilization screen of the vegetable oils revealed the best growth on SW medium containing maize oil with sole carbon source. The maximum values for the biosurfactant concentration obtained in liquid medium containing 1 % of maize oil was to 200 mg l^{-1} for *P. aeruginosa*, 56.55 mg l^{-1} for *P. fluorescens* and 23.45 mg l^{-1} for *P. putida* after 120 h, during to exponential phase. The characterization of the biosurfactant produced was performed by thin layer chromatography and showed the presence of two spots with R*f* values equal 0.73 and 0.47. The strain *P. aeruginosa* distinguished from the strains *P. fluorescens* and *P. putida* by major adaptation to the produced biosurfactant on vegetal oil in low concentration of nitrogen and micronutrients.

Keywords: Biosurfactant, Maize oil, *Pseudomonas*

*Corresponding author. Tel.: (82) 32141380;
Fax: (82)32141615; E-mail: amql@qui.ufal.br

1. Introduction

Biosurfactants are amphiphilic molecules consisting of hydrophilic and hydrophobic domains. These compounds are capable of reducing surface and interfacial tension among liquids, solids and gases, allowing them to mix or disperse readily as emulsions, in water or other liquids [1]. The agent surface tensoative have many advantages in comparison to the synthetic surfactants, such as: low toxicity, biodegradability, great chemical and structural diversity [2]. Research in the biosurfactants area has expanded quite a lot in recent years due to its potential use in different areas, such as the food industry, paper and pulp industry among others, agriculture, pharmaceutics, oil industry, petrochemistry, [3].

Pseudomonas species, ubiquitous in soil and water, are of considerable scientific and technological importance and comprise a taxon of metabolically versatile organisms capable of utilizing a wide range of simple and complex organic compounds [4]. Rhamnolipids are compounds produced by *Pseudomonas aeruginosa* which reduce water surface tension and emulsify oil [5]. The synthesis of rhamnolipids proceeds by sequential glycosyl transfer reactions, each catalysed by a specific rhamnosyltransferase with TDP-rhamnose acting as a rhamnosyl donor and 3-hydroxydecanoyl-3-hydroxydecanoate acting as the acceptor.L-Rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate and L-Rhamnosyl- 3-hydroxydecanoyl-3-hydroxydecanoate, referred to as rhamnolipids 1 and 2, respectivel. They are the principal glycolipids produced in liquid cultures [6]. Rhamnolipid surfactants can be produced from various substrates, typically renewable resources such as vegetable oils and distillery and dairy wastes. Vegetable oils and residues from vegetable oil refinery are among the most used low-cost substrates for rhamnolipids production, whereas accumulation from glucose and hydrophilic carbon sources generate lower yields and are extensively explored [7].

The study of the production of biosurfactants by pseudomonads during many years was focused in the research of rhamnolipids produced by *P. aeruginosa* [8-9-10-11]. The production of a glycolipid in the form of a rhamnolipid was obtained by the growth of *Pseudomonas fluorescens* (NCIMB 11712) on virgin olive oil [12]. Recently was reported the biosurfactant production by strain *Pseudomonas putida* 21BN on soluble substrates, such as glucose or on poorly soluble substrates, such as hexadecane, presented its chemical nature as rhamnolipídic [13]. On the other hand, Bonilla *et al*. [14] identified the bioemulsifier produceted by a nonfluorescent strain of *Pseudomonas putida ML2* as polysaccharidic nature of the biosurfactant. This is due to the great metabolic versatility presented by the different ones species and strains of *Pseudomonas*. The objective of this work was to verify the production of biosurfactant

in continues culture containing maize oil by *Pseudomonas* species isolated from agri-industrial effluent.

2. Material and methods

2.1. Isolation of bacteria from agri-industrial effluent

Samples of effluent caming from sugar cane laudering of the S.A. Coruripe agri-industrial where was collected and carried to the Biochemist Laboratory of the Vegetable Parasitism and Ambient Microbiology (Federal University of Alagoas/Brazil). During isolation 1 ml of the water sample was transferred asseptically into a 500 ml Erlenmeyer flask containing 100 ml water sterile. Aliquot of 0.1 ml was spread on medium MCC plates containing (g l^{-1}): peptone, 5.0; meat extract, 3.0; NaCl, 1.0; agar, 15.0 and 25 % sugar cane broth; pH adjusted to 7.0. Colonies morphologically different that appeared after three days of incubation were transferred to plates containing medium MCC.

2.2. Screening of biosurfactant producing isolates

Bacterial strains were initially assayed for rhamnolipid production using the mineral salt-CTAB-methylene blue agar plate method originally developed by Siegmund and Wagner [15]. SW medium was prepared adding 0.2 g of CTAB and 0.005 g of methylene blue to 1 l of mineral medium containing (g l^{-1}) glycerol, 20.0; KH_2PO_4, 0.7; Na_2PO_4, 0.9; $NaNO_3$, 2.0; $Mg_2SO_4 7 \cdot H_2O$, 0.4; $CaCl_2 \cdot H_2O$, 0.1 and 2 ml mineral solution containing (g l^{-1}): $FeSO_4.7H_2O$, 1.0; $MnSO_4.H_2O$, 1.5; $(NH_4)_2MnO_7.4H_2O$, 0.6; agar, 15.0. The plates were incubated at 30 ^{0}C for 72 h. Positive reaction for rhamnolipids was visualized by formation of a dark blue halo with a sharply defined around the colonies. Canola, sunflower, maize oils were used as carbon source as substitute of the glycerol. The plates were inoculated with approximately 1 x 10^3 cells ml^{-1} and incubated at 30 ^{0}C for 72 h. The growth was determined in terms of c.f.u. ml^{-1}. The vegetable oils were obtained commercially.

2.3. Identification of strains selected

The phenotypic characterization of the selected isolates was based on Gram staining, cell morphology and classical biochemical tests (oxidase “Kovacs”, nitrate “to nitrite”, catalase, test for indole, urease, citrate utilization, oxidation and fermentation of glucose) following the Bergey’s Manual [16]. The commercial kit API 20E

(BioMérieux, France) was used to identify the isolates that presented positive results in the glycolipidic biosurfactant production in SW medium.

2.4. Growth conditions

The growth medium used for production of rhamnolipid was (g l^{-1}): yeast extract, 0.1; NaCl, 1.0; $MgSO_4$, 0.2; $MnSO_4.H_2O$, 0.02. Finally, 1 % of maize oil (w/v) was added. The fermentation process was carried out in 1000 ml Erlenmeyer flasks, containing 300 ml of medium. The medium was sterilized at 120 ^{0}C, 1 atm for 20 min. The final pH of medium was adjusted to 7.0. The concentration of inoculum consisting of 1 x 10^3 cells ml^{-1}. Submerged microbial cultures were incubated at 30 ^{0}C at 150 rpm for 120 h. Aliquots of 10 ml of the culture were withdraw each 24 h for pH measurement, for determination of the glycolipid concentration and cell growth evaluation by absorbance at 600nm (OD_{600}).

2.5. Determination of Glycolipid(Rhamnolipid) production

The glycolipids concentration was determined in triplicate by using orcinol assay [17]. To 0.1 ml of each sample from the cells culture supernatant 0.9 ml of a solution containing 0.19 % orcinol (in 53 % H_2SO_4) was added, after that heating for 30 min at 80 ^{0}C. Then the samples were cooled for 15 min at room temperature and the A_{421} was measured. The glycolipid concentration was evaluated based on a standard curves prepared with L-rhamnose and converts this to the rhamnolipid concentration.

2.6. Glycolipid characterization by thin-layer chromatography

The cell culture supernatant was concentrated by lyophilization. The concentrate was treated twice with $CHCl_3$:MeOH (2:1, v/v). The solvents were removed by evaporation. The material was dissolved in $CHCl_3$:H_2O (1:1, v/v) and analysed by thin-layer chromatography (TLC) on silica gel plates (G60; Merck, Germany). Chromatograms were developed with $CHCl_3$:CH_3OH:H_2O (65:25:4, v/v/v) as eluent and visualized with different reagents, i.e. ninhydrin for free amino groups, iodine vapours and rhodamine 6G for lipid strain, bromocresol-green for free carboxylic acid and Molish reagent for sugar detection [18].

3. Results and discussion

3.1. Screening of rhamnolipid production

The production of rhamnolipids by bacterial cells was initially used as criterion of selection. The bulk of halo is proportional to the quantity of rhamnolipids produced by different isolates from analyzed agri-industrial effluent [15]. The C1 and C3 isolates showed halos around their colonies on SW medium plates. The halo of the C1 and C3 isolates were 2.4 cm and 0.97 cm, respectively. However, the C2 isolate, presented growth in SW medium without formation of halo, but the colony diameter was equal 1.0 cm. The C1 isolate was the species that presented the biggest halo corresponding to the biosurfactant production (Figure 1). Similar observations were reported by Perfumo *et al.* [19] that evaluated the production of anionic glycolipid biosurfactants in SW medium by *P. aeruginosa* AP02-1 isolated from a hot spring environment. The AP02-1 strain grew forming colonies surrounded by halos major 2.0 cm diameter, after 24 h of incubation at 45 ^{0}C.

The influence of carbon source on the growth of different bacterial isolates was evaluated in SW medium containing canola, sunflower and maize oils. Maize oil was found to be the best substrate for multiplication of C1 isolate and C3 isolate, as the highest cell count of 7 x 10^{8} c.f.u. ml^{-1} for C1 isolate and 6 x 10^{7} c.f.u. ml^{-1} for C3 isolate were obtained from an initial count of 1 x 10^{3} c.f.u. ml^{-1} with 72 h of incubation (Table 1). The composition of free fatty acids present in maize oil containing in major quantities of linoleic acid, oleic and palmitic acids that can be utilized as substrate for microbial growth a biosurfactant production. In general the use of vegetal oils as carbon source in the synthesis of biosurfactants present advantages over carbohydrates, therefore they are carbon sources of low cost and do not have toxic effect on the cells [20].

3.2. Identification of strain selection

Among 15 bacterial isolates from agri-industrial effluent, three species of *Pseudomonas* were found to efficiently utilize vegetable oils as carbon source from growth and produce biosurfactant. Detailed biochemical characterization of the isolates showed (Table 2) that they were three different *Pseudomonas* isolates. On the basis of the biochemical results, morphologic characteristics together with the commercial kit API 20E (Table 2) was possible to identify three different *Pseudomonas* species: *P. aeruginosa* (C1 isolate), *P. fluorescens* (C2 isolate) and *P. putida* (C3 isolate).

3.3. Production of rhamnolipid in maize oil medium

The isolates of *P. aeruginosa*, *P. fluorescens* and *P. putida* were compared for possible biosurfactant producing ability by cultivation in medium containing maize oil as the carbon source, at 30 ^{0}C for 120 h of incubation (Figure 2). The maximum concentrations of the biosurfactant at 120 h after the inoculation obtained were for *P. aeruginosa* isolate around 200 mg l^{-1}, of 56.55 mg l^{-1} for *P. fluorescens* and 23.45 mg l^{-1} for *P. putida*, during exponential phase. The optical density OD_{600} and biosurfactant glycolipidic production increased gradually in parallel for five days incubation. The results showed that the production did not present any drastic alteration of pH.

These results are in accordance with previous observation done by Patel & Desai [21] that evaluated the use of renewable water soluble byproducts in the biosurfactant production. After 96 h the culture supernatant fluid had an equivalent rhamnose content of 240 mg l^{-1}. Similar observations were reported by Santa Anna *et al*. [22] that evaluated the production of biosurfactant by *P. aeruginosa* strain PA1 cultivated during seven days fermentation period in culture medium with the following composition (g l^{-1}): $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.2; using babassu oil as carbon source. The maximum concentration of rhamnolipid after 144 h of fermentation was equal 200 mg l^{-1}.

3.4. Characterization of the biosurfactant obtained

The biosurfactant extracted from the culture supernatant concentrate was developed by TLC and visualized with specific reagents and produced spots with different R*f* (retention factor) values (Figure 3). The spots with R*f* 0.73 and Rf 0.47 showed positive reactions for sugars with Molish reagent and for lipids with rhodamine B reagent, but negative reactions for amino groups with ninhydrin and for UV (Table 3). These results suggest that *Pseudomonas* species produces two glycolipidic biosurfactant in maize oil medium.

These results are in good agreement with Haba *et al.* [23] who observed that *Pseudomonas* sp when cultivated in medium containing vegetable oils, produced two types of rhamnolipids with R*f* of 0.7 and 0.45, respectively. Similarly, the biosurfactants RL1 and RL2 were purified by chromatography on a column of silica gel 60 using the solvent system $CHCl_3$:MeOH:AcOH (65:15:2, v/v/v). The localization of spots was performed with a thymol spray reagent and R*f* values of RL1 and RL2 were 0.74 and 0.36, respectively [24].

This observation is supported by results obtained by Arino *et al.* [25] that characterized by chromatography the mixture rhamnolipid produced by *P. aeruginosa*

GL1. The R*f* value for different spots were calculed and it corresponds at to R1 0.72 (1 rhamnose and 2 fatty acids); R2 0.40 (1 rhamnose and 1 fatty acids); R3 0.32 (2 rhamnose and 2 fatty acids); and R4 0.13 (2 rhamnose and 1 fatty acids). In particular, the rhamnolipidic biosurfactant produced by *P. aeruginosa* AT10 in cultures of soybean oil refinery waste, was often identified a mixture of up to seven homologues (R2C10C10 + R1C10C10 + R2C10C12 + R1C10C12 + R1C12:1C10 + R1C12:2 + R1C8:2) [26]. It is well known that there are different structural variants of rhamnose lipids; the type produced depends on the *Pseudomonas* strain, the carbon source used and the strategy of production as well [7].

These results indicate that *P. aeruginosa* secretes efficient oil/fatty acid inducible rhamnolipidic biosurfactant degrading maize oil. That it would be very valuable not only for industrial applications, but also for disposal of problematic waste oils.

Acknowledgements

This work was financially supported by the Finep/CTHidro; BNB and S.A. Coruripe Sugar and Alcohol.

References

[1]Vance-Harrop, M.H., Gusmão, N.B., Takaki, G.M.C. (2003) New bioemulsifiers produced by *Candida lipolytica* using D-glucose and babassu oil as carbon sources. Brazilian J. Microbiol., 34, 120-123,

[2]Kosaric, N. (1992) Biosurfactants in industry. Pure Appl. Chem., 64:1731-1737.

[3]Sarney, D.B. and Vulfson, E.N. (1995) Application of enzymes to the synthesis of surfactants. Tibtech, 13, 164-172.

[4]Hasanuzzaman, M., Umadhay-Briones, K.M., Zsiros, S., Morita, N., Nodasaka, Y., Yumoto, I. and Okuyama, H. (2004) Isolation, Identification, and Characterization of a Novel, Oil-Degrading Bacterium, *Pseudomonas aeruginosa* T1. Current Microbiol., 49, 108-114.

[5]Wild, M., Caro, A.D., Hernández, A.L., Miller, R.M. and Soberón-Chávez, G. (1997) Selection and partial characterization of a *Pseudomonas aeruginosa* mono-rhamnolipid deficient mutant. FEMS Microbiol. Lett., 153, 279-285.

[6]Burger, M.M., Glaser, L. and Burton, R.M. (1963). The Enzymatic Synthesis of a Rhamnose-containing glycolipid by extracts of *Pseudomonas aeruginosa*. T. J. Biolog. Chemist., 238, 2595-2602.

[7]Nitschke, M., Costa, S.G.V.O. and Contiero, J. (2005) Rhamnolipid surfactants: An update on the general aspects of these remarkable biomolecules. Biotecnol. Prog., 25, 1593-1600.

[8]Robert, M., Mercadé, M.E., Bosch, M.P., Parra, J.L., Espuny, M.J., Manresa, M.A. and Guinea, J. (1989) Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T1. Biotechnol. Lett., 11, 871-874.

[9]Ochsner, U.A. Reiser, J., Fiechter, A., and Witholt, B. (1995) Production of *Pseudomonas aeruginosa* rhamnolipid biosurfactants in heterologous host. Appl. Environm. Microbiol., 61, 3503-3506.

[10]Noordman, W.H., and Janssen, D.B. (2002) Rhamnolipid stimulates uptake of hydrophobic compounds by *Pseudomonas aeruginosa*. Appl. Environm. Microbiol., 68, 4502-4508.

[11]Raza, Z.A., Rehman, A., Khan, M.S. and Khalid, Z.M. (2007) Improved production of biosurfcatnt by *Pseudomonas aeruginosa* mutant using vegetable oil refinery waste. Biodegradation, 18, 115-121.

[12]Healy, M.G., Devine, C.M. and Murphy, R. (1996) Microbial production of biosurfactants. Resour. Conserv. Recycling, 18, 41-57.

[13]Tuleva, B.K., Ivanov, G.R. and Christova, N.E. (2002) Biosurfactant production by a new *Pseudomonas putida* strain. Zeitschrift Fur Naturforschung C: J. Biosci. 57**,** 356–360.

[14]Bonilla, M., Olivaro, C., Corona, M., Vazquez, A. and Soubes, M. (2005) Production and characterization of a new bioemulsifier from *Pseudomonas putida* ML2. J. Appl. Microbiol., 98, 456-463.

[15]Siegmund, I. and Wagner, F. (1991) New method for detecting rhamnolipds excreted by *Pseudomonas* species during growth on mineral agar. Biotecnol. Techniq., 5, 265-268.

[16]Holt, J.G., Krieg, N.R., Sneath P.H.A., Staley J.T, Williams, S.T. (1999) Bergey's Manual of Determinative Bacteriology. 9ª edition. Williams and Wilkins, (ISBN 0-683-00603-7).

[17]Chandrasekaran, E.V. and Bemiller, J.N. (1980) Constituent analysis of glycosaminoglycans. In: Whistler, R.L., Wolfrom, M.L (Eds.), Methods in Carbohydrate Chemistry. Academic Press, New York, p. 89-96.

[18]Parra, J.L., Guinea, J., Manresa, M.A., Robert, M., Mercadé, M.E., Comelles, F. and Bosch, M.P (1989) Chemical characterization and physicochemical behavior of biosurfactants. JAOCS, 66, 141-145.

[19]Perfumo, A., Banat, I.M., Canganella, F. and Marchant, R. (2005) Rhamnolipid production by a novel termophilic hydrocarbon degrading *Pseudomonas aeruginosa* API2-1. Appl. Microbiol. Biotechnol., 72, 132-138.

[20]Shabtai, Y. and Wang, D.I.C. (1990) Production of emulsan in a fermentation process using soybean oil (SOB) in a carbon nitrogen coordinated feed. Biotechnol. Bioeng., 35, 753-765.

[21]Patel, R.M. and Desai, A.J. (1997) Biosurfactant production by *Pseudomonas aeruginosa* GS3 from molasses. Lett. Appl. Microbiol., 25:91-94.

[22]Santa Anna, L.M., Sebastian, G.V., Menezes, E.P., Alves, T.L.M., Santos, A.S., Pereira JR.N. and Freire, D.M.G (2002). Production of biosurfactants from *Pseudomonas aeruginosa* PA1 isolated in oil environments. Brazilian J. Chem. Engineer., 19,159-166.

[23]Haba, E., Espuny, M.J., Busquets, M. and Manresa, A. (2000) Screening and production of rhamnolipids by *Pseudomonas aeruginosa* 47T2 NCIB 40044 from waste frying oils. J. Appl. Microbiol., 88: 379-387.

[24]Schenk, T., Schuphan, I. and Schmidt, B. (1995) Higth-performance liquid chromatographic determination of the rhamnolipids produced by *Pseudomonas aeruginosa*. J. Chromatogr. A, 687, 7-13.

[25]Arino, S., Marchal, R. and Vandecasteele, J.P (1996) Identification and production of rhamnolipdic biosurfactant by a *Pseudomonas* species. Appl. Microbial Biotechnol., 45,162-168.

[26]Abalos, A., Pinazo, A., Infante, M.R., Casals, M., García, F. and Manresa, A. (2001) Physicochemical and antimicrobial properties of new rhamnolipids produced by *Pseudomonas aeruginosa* AT10 from soybean oil refinery wastes. Langmuir, 17, 1367-1371.

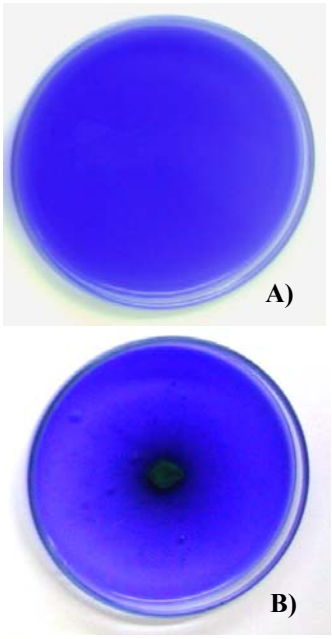

Fig.1. Dark blue halo around the colony in SW medium associated with glycolipidic biosurfactant production. A: plate containing SW medium in absence of microorganisms, and B: plate containing SW medium inoculated with C1 isolated after 72 h of incubation at 30^0C.

Table 1
Screening of different carbon source for biosurfactant production in SW medium containing maize, canola and sunflower oils by isolates obtained from agri-industrial effluent.

Type of vegetable oil	Growth (c.f.u. m l^{-1})			
	0 h	C1 Isolate (72 h)	C2 Isolate (72 h)	C3 Isolate (72 h)
*Maize oil	1 x10^3	7 x10^8	2 x10^7	6 x10^7
Canola oil	1 x10^3	5 x10^8	2 x10^7	2 x10^7
Sunflower oil	1 x10^3	3 x10^8	1 x10^7	1 x10^7

*Maize oil (1 %; w/v) was screened for biosurfactant production as a sole source of carbon in medium poor in nutrient. The study was performed in triplicate.

Table 2
General characteristics of different isolates from agri-industrial effluent.

Characteristics	**C1**	**C2**	**C3**
Cell Morphology			
Gram staining	Gram -	Gram -	Gram -
Motility	Highly motile	Highly motile	Highly motile
Sporulation	Absence	Absense	Absence
Cultural			
Mac Conkey	+	+	+
Pyocyanin* (King's-A medium)	+	-	-
Fluorescein* (King's-B medium)	+	+	+
Pyorubin* (Nutrient agar)	+	-	-
Growth at 42°C	+	-	-
Growth at 4°C	-	+	-
Growth at NaCl 6,5%	+	-	-
Growth on Citrate	-	+	+
Production of Acetoine	-	-	-
Production of Indole	-	-	-
Production of H_2S	-	-	-
NO_2	-	-	-
N_2	+	-	+
Biochemical			
Oxidation/Fermentation			
Glucose	+	-	-
Manitol	-	-	-
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Sucrose	-	+	-
Melibiose	-	-	-
Amigdaline	-	-	-
Arabinose	-	-	-
Enzyme production			
Catalase	+	+	+
Oxidase	+	+	+
β-galactosidase	-	-	-
Arginine Dihydrolase	+	-	+
Lisine Descarboxilase	-	-	-
Ornitine Descarboxilase	-	-	-
Triptofan Deaminase	-	-	-
Citocrome Oxidase	+	+	+
Lipase	+	+	+
Gelatinase	+	-	-
Amylase	+	+	+
Urease	+	-	-
Caseinase	+	-	-
Proteinase	+	+	+
DNAse	+	+	+
Polygalacturonase	-	-	-
Pectate trans-eliminase	-	-	+

*Type of pigment
- Negative result
+ Positive result

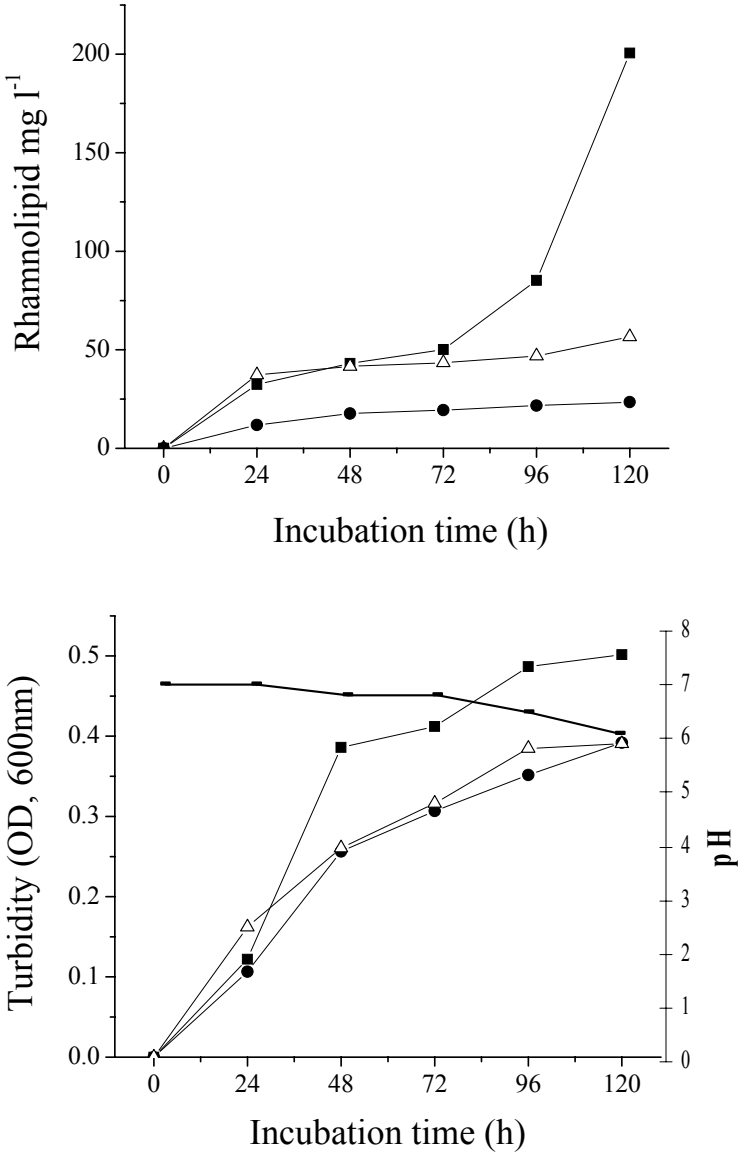

Fig. 2. Production of biosurfactant (glycolipids) by isolates *Pseudomonas* in medium containing 1 % maize oil. Incubation was done at 30 ^{0}C at 150 rpm. The growth was expressed in Optical Density (OD_{600}) and biosurfactant levels in rhamnolipids mg l^{-1}. *P. aeruginosa* (■), *P. fluorescens* (Δ) and *P. putida* (●). Values are averages from three cultures.

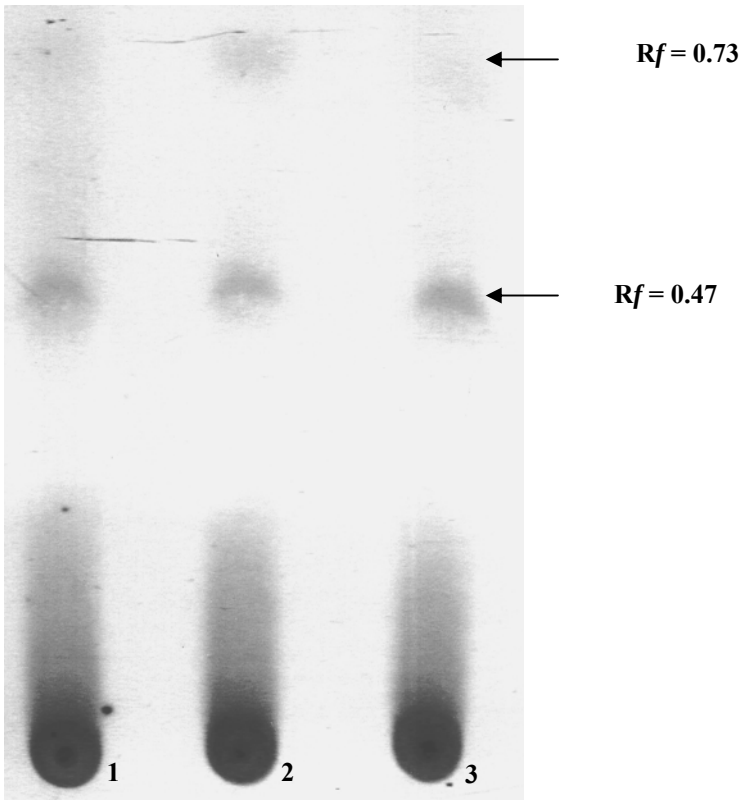

Fig. 3. Rhamnolipid components detected by thin layer chromatography. Lane 1, *Pseudomonas fluorescens*, line 2 *Pseudomonas aeruginosa* and line 3 *Pseudomonas putida*. The samples were applied in 20 µl volumes in TLC plates (26 cm x 3.5 cm silica gel column) and developed with $CHCl_3$:MeOH:H_2O (65:25:2) solvent system. TLC plate was sprayed with Molisch reagent, followed by heating at 100^0C for 10 min. Carbohydrate remained in the origin.

Table 3
Thin layer chromatography parameters of the different forms of glycolipids.

Spots	Rf	[a]UV	[b]Ninhydrin	[c]Bromocresol green	[d]Rhodamine 6G	[e]Iodine vapours	[f]Molish
1	0.73	-	-	+	+	+	+
2	0.47	-	-	+	+	+	+

Solvent system CH_3Cl:CH_3OH:H_2O (65:25:4)
[a]UV: Detection of various compounds which have native fluorescence
[b]Ninhydrin: Detection of free amino groups
[c]Bromocresol green: Detection of free carboxylic acid
[d]Rhodamine 6G: Detection of lipids
[e]Iodine vapours: Detection of many organic compounds
[f]Molish: Detection of sugar

6. CONCLUSÕES

Com base nos resultados obtidos, foi possível concluir que:

1. Três bactérias Gram negativas (C1, C2 e C3) obtidas a partir de efluente agroindustrial apresentaram crescimento em meio SW para a produção de ramnolipídios;
2. Com base nas características morfoculturais e bioquímicas, juntamente com os resultados obtidos pelo uso do kit comercial API20E foram identificadas três diferentes espécies de *Pseudomonas*: *P. aeruginosa*, *P. fluorescens* e *P. putida*;
3. O óleo de milho consistiu no melhor substrato para o crescimento de *P. aeruginosa* e *P. putida* sob meio SW, sendo utilizado em estudos em meio líquido;
4. A produção de biosurfactante glicolipídico em meio liquido contendo 1 % de óleo de milho foi detectada para todos os isolados, entretanto *P. aeruginosa* apresentou maior rendimento;
5. O isolado *P. aeruginosa* apresentou potencial uso em estudo de biorremediação de contaminantes com baixa solubilidade em água.

7. PERSPECTIVAS

- Caracterizar filogeneticamente os isolados obtidos pela amplificação da subunidade 16S do rRNA genômico por PCR, usando o par universal de iniciadores para bactérias, 27F e 1522R, comparando-os com espécies do mesmo gênero provenientes da Coleção de Culturas Tropical (CCT) da Fundação André Tosello (Campinas/SP);

- Otimizar a produção de biosurfactantes sob meios de cultura economicamente viáveis, como por exemplo subprodutos industriais;

- Extrair e caracterizar os biosurfactantes obtidos no melhor meio indutor desse produto, visando compreender melhor as vias utilizadas pelos isolados selecionados para sua síntese;

- Estabelecer as condições nutricionais e físico-químicas onde a produção do biosurfactante pelos diferentes isolados de *Pseudomonas* se mostre mais eficaz.

ANEXOS

➢ Trabalhos apresentados em congressos

- ✓ PEREIRA, D. S. T., SILVA, Amanda Lys Santos, PEROVANO FILHO, Natalino, SILVA, Maria da Paz Carvalho, LOPEZ, Ana Maria Queijeiro Produção de biosurfactante por linhagens de *Pseudomonas putida*. In: III workshop de biocatálise e II encuentro regional de biocatalisis e biotransformaciones, 2006, São Paulo/SP, Brasil.

- ✓ PEREIRA, D. S. T., SILVA, Amanda Lys Santos, SANTANA FILHO, Arquimedes Paixão, PEROVANO FILHO, Natalino, SILVA, Maria da Paz Carvalho, LOPEZ, Ana Maria Queijeiro. Production of biosurfactant and extracellular enzymes by differents strains of species of *Pseudomonas*. In: XXXV Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2006, Águas de Lindóia/SP, Brasil.

- ✓ PEREIRA, D. S. T., SILVA, Amanda Lys Santos, Maria da Paz Carvalho, LOPEZ, Ana Maria Queijeiro. Evaluation of the growth of *Pseudomonas* different species on lubricant oil In: VIII[a] Reunião Regional da SBBq, Universidade Federal do Rio Grande do Norte, 2006, Natal/RN, Brasil.

- ✓ PEREIRA, D.S.T., SILVA, Amanda Lys dos Santos, PEROVANO FILHO, N., SILVA, Kelly Fernanda Seara, SILVA, Nicácio Henrique[2]; SILVA; Maria da Paz Carvalho[2] & LÓPEZ, Ana Maria Queijeiro. Influences of the carbon source in the production of glycolipidic biosurfactant for *pseudomonas* spp. Submetido In: XXXVI Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2007, Salvador/BA, Brasil.

➢ Instruções de autores dos periódicos

- ✓ FEMS Microbiology Letters

Trabalhos apresentados em congressos

PRODUCTION OF BIOSURFACTANT AND EXTRACELLULAR ENZYMES BY DIFFERENTS STRAINS OF SPECIES OF *Pseudomonas*

Pereira, D.S.T.[1,2]; Silva, A.L.S.[1]; Santana Fº, A. P[1].; Perovano Fº, N. [1]; Silva, K.F.S. [1]; Santos, E. S.[1]; Silva; M.P.C.[2] & López, A.M.Q.[1]

[1]Universidade Federal de Alagoas, Instituto de Química e Biotecnologia, Lab. de Bioq. do Parasitismo Vegetal e Microbiologia Ambiental, BR 104 N, Km 97, Maceió/AL, CEP 57072-970, amql@qui.ufal.br ; [2]Universidade Federal de Pernambuco, CCB, Depto de Bioquímica

The biosurfactants have many advantages in comparison to the sinthetic surfactants, such as low toxicity, biodegradability, and chemical and structural diversity. The purpose of this work was to study the application of the method of the oil spreading to quantify the production of surfactants by different strains of *Pseudomonas* [*P. putida, P. aeruginosa, P. fluorescens*, and three isolates of *Pseudomonas* spp. (Iso 1, Iso 2 and Iso 3)], and also to verify the extracellular secretion of some enzymes (amylases, pectinases, nucleases, proteases and lipases) of the same microorganisms. The composition of the growth medium used in the oil spreading method was (g L^{-1}): 2.7 KH_2PO_4; 13.9 K_2HPO_4; 10.0 sacarose; 50.0 NaCl; 0.5 yeast extract; 1.0 $NaNO_3$; after the autoclavation of this, it was added the follow autoclaved aqueous solutions (10 mL L^{-1}) A (25.0 gL^{-1} $MgSO_4$) and B [100.0 gL^{-1} $(NH_4)_2SO_4$], and the filtered (0.22 □m) solution C (gL^{-1}: 0.5 EDTA; 3.0 $MnSO_4.H_2O$; 1.0 NaCl; 0.1 $CaCl_2.2H_2O$; 0.1 $ZnSO_4.7H_2O$; 0.1 $FeSO_4.7H_2O$; 0.01 $AlK(SO_4)_2$; 0.01 $Na_2MoO_4.2H_2O$; 0.01 H_3BO_3; 0.00001 $CuSO_4.5H_2O$). This is a modified composition of the medium described by Yossef *et al.*(2004). Then, the optical density $_{600nm}$, and the number of cells mL^{-1} were determined at intervals of 24 h, during 120 h. Aliqüotes of the filtered growth medium were also analysed by the mineral oil spreading method, after the construction of a standard courve using different concentrations (0-1000 mgL^{-1}) of a sinthetic surfactant (Duodecil Sulfate of Sodium - SDS) *versus* the diameter of the degradation of mineral oil. The maximum concentrations of the biosurfactant 120 h after the inoculation were 247 eq. mg of $SDSL^{-1}$ for Iso 1; 180 eq. mg of $SDSL^{-1}$ for Iso 2, 178 eq. mg of $SDSL^{-1}$ for *P. putida*; 170 eq. mg of $SDSL^{-1}$ for *P. fluorescens*; 166,2 eq. mg of SDS L^{-1} for *P. aeruginosa;* and 148,4 eq. mg of SDS L^{-1} for Iso 3. The increase of the optical density was parallel to the surfactant production during the incubation time. Regarding the tests using different substrates in solid growth medium, 72 h after the incubation (30 ± 1 ºC, dark), all the strains produced the analyzed enzymatic activities. Then, the tested strains of *Pseudomonas*, mainly the Iso 1, showed potencial to be used in combination with other microorganisms in assays of bioremediation of industrial effluents containing liposoluble compounds.

Financial Support: Finep/CTHidro; BNB; Capes; CNPq; Fapeal; S.A. Usina Coruripe Açúcar e Álcool.

EVALUATION OF THE GROWTH OF *Pseudomonas* DIFFERENT SPECIES ON LUBRICANT OIL

DANIELLE, S. T. PEREIRA[1; 2]; AMANDA LYS S. SILVA[2]; MARIA DA PAZ C. SILVA[1]; ANA MARIA Q. LÓPEZ[2]

[1]Universidade Federal de Pernambuco; [2]Universidade Federal de Alagoas.

The great metabolic diversity of the *Pseudomonas* genus makes possible the use of these microorganisms on the ambient contaminations treatment. The intention of this work was to evaluate the growth of the different species of *Pseudomonas* on lubricant oil and verifying the extracellular production of rhamnolipid biosurfactant. Strains of *P. putida*, *P. aeruginosa* and *P. fluorescens*, were inoculated in solid medium containing 1 % lubricant oil, 0.1 % NaCl and 0.1% of one of the three different nitrogen sources [yeast extract, $NaNO_3$ and $(NH_4)_2SO_4$]. The production of the biosurfactant was verified in medium described by Siegmund & Wagner (1991), which is constituted of KH_2PO_4; Na_2HPO_4; $NaNO_3$; $MgSO_4$; $CaCl_2$; Agar; $FeSO_4$; $MnSO_4$; $(NH_4)_6MnO_7$; CTAB and of methylene blue. From the evaluated species, *P. putida* showed a growth reduction in a medium containing $NaNO_3$ and $(NH_4)_2SO_4$, while *P. fluorescens* showed only a growth reduced when cultivated under $NaNO_3$ as nitrogen sources. On the other hand, *P. aeruginosa* did not presented any variation of growth by using the tree different studied nitrogen sources, being such species the one that secreted the highest concentration of rhamnolipid. The results point out the potential use of *P. aeruginosa* bioremediation studies of petroleum derivatives.

Supported by: CAPES, BNB; Usina Coruripe Açúcar e Álcool, UFPE.

Key words: *Pseudomonas*, biosurfactant; bioremediation

*INFLUENCES OF THE CARBON SOURCE IN THE PRODUCTION OF GLYCOLIPIDIC BIOSURFACTANT FOR *Pseudomonas* spp

Pereira, D.S.T.[1,2]; Silva, A.L.S.[1]; Perovano Fº,N. [1]; Silva, K.F.S. [1]; Silva, N.H.[2]; Silva; M.P.C.[2] & López, A.M.Q.[1]

[1]Universidade Federal de Alagoas, Instituto de Química e Biotecnologia, Lab. de Bioquímica do Parasitismo Vegetal e Microbiologia Ambiental, amql@qui.ufal.br;
[2]Universidade Federal de Pernambuco, CCB, Depto. de Bioquímica

Biosurfactants are amphiphilic compounds of large importance industrial. The purpose of this work was to verify the production of biosurfactants by different strains of *Pseudomonas* (C1, C2 and C3) isolated of effluent agri-industrial, having maize oil as carbon source. The composition of the growth medium used was (g/L): 2 $MgSO_4$; 3 $MnSO_4$. H_2O; 0.1 $FeSO_4$.7 H_2O; 0.1 NaCl; 0.1 yeast extract and 1% maize oil. Aliqüots of the filtered growth medium were utilized for measure the pH, orcinol assay and emulsifying unit. The different glycolipidic types were separated by TLC using $CHCl_3$:CH_3OH:H_2O (65:25:4, v/v/v) as solvent system. On the basis of the biochemist results (commercial kit API 20E) were possible identify three different *Pseudomonas* species: *P. aeruginosa*, *P. fluorescens* and *P. putida*. The maximum concentrations of the biosurfactant obtained were 200.58 mg/L *P. aeruginosa*, 56.57 mg/L *P. fluorescens* and 23.45 mg/L *P. putida*. The emulsifying unit 120h after the inoculation was 2.013 *P. aeruginosa,* 0.8 *P. fluorescens* and 0.192 *P.putida.* The TLC analyses produced spots with different values R_f (0.73 and 0.47) showing positive reactions for sugars with Molish reagent. The *P.aeruginosa* species showed potential to be used in consortium with other microorganisms in assays of bioremediation.

Keywords: Biosurfactant, Maize oil, *Pseudomonas*, Thin layer chromatography

Financial Support: Finep/CTHidro; BNB; Capes; CNPq; Fapeal; S.A. Usina Coruripe Açúcar e Álcool, UFPE.

*Resumo submetido avaliação da comissão organizadora da XXXVI Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2007, Salvador/BA, Brasil.

Instruções de autores dos periódicos

ELSEVIER

FEMS Microbiology Letters 242 (2005) I–IX

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Notes to Authors

FEMS Microbiology Letters

AIMS AND SCOPE

The Editors give priority to concise papers that merit urgent publication by virtue of their originality, general interest and their contribution to new developments in microbiology. All aspects of microbiology, except virology (other than bacteriophages), are covered. Areas of special interest include: molecular biology and genetics; genomics, microbial biochemistry and physiology; structure and development; pathogenicity, medical and veterinary microbiology; environmental microbiology; applied microbiology and microbial biotechnology; systematics. Papers can deal with any sort of microorganisms: bacteria and bacteriophage, yeasts, filamentous fungi and protozoa. Papers should be complete in themselves and adequately supported by experimental detail; they should not be preliminary versions of communications to be published elsewhere.

Descriptions of new methods are acceptable, and the Editors are also prepared to consider papers that put forward new hypotheses. However, papers that provide confirmatory evidence or merely extend observations firmly established in one species to another will not be accepted unless there are strong reasons for doing so.

MINIREVIEWS

MiniReviews are concise articles covering topics of current interest or controversial aspects of subjects within the scope of the journal. The style for MiniReviews is the same as for Research Letters with the following amendments: the maximum length of the text is about 12 double-spaced manuscript pages (3500 words); a combined total of six figures and tables is allowed. The number of references should normally not exceed 50. Colour figures or diagrams are encouraged and will be printed in the journal free of charge providing the Editor believes that the use of colour adds value to the MiniReview. There is no rigid format for MiniReviews but they should generally include an Abstract and a brief Introduction in which the background to the article is presented. It is then helpful if the remainder of the text is arranged under a single, or a maximum two levels of subheading, finishing with a Conclusion or Outlook section.

MiniReviews are normally invited, but prospective authors are encouraged to contact the MiniReviews Editors, Rustam I. Aminov, Ian Henderson or Richard C. Staples, to discuss possible contributions. Their address details can be found in the front pages of each issue and on the Journal webpage. An account for online submission will be created for authors invited to write a MiniReview.

LETTERS TO THE EDITOR

Letters to the Editor are brief communications focusing on a Research Letter or a MiniReview that has been published in this journal within the previous six months. They should focus on some aspect(s) of the paper that is, in the author's opinion, incorrectly stated or interpreted, controversial, misleading or in some other way worthy of comment. All Letters to the Editor must address a scientific issue in an objective fashion, should be fewer than 1000 words, and will be externally refereed. If acceptable for publication, they will be offered to the original authors for comment. Please choose the manuscript type "Letter to the Editor" when uploading this type of Letter to the online submission system.

SUBMISSION PROCEDURE

Manuscripts should be submitted through Manuscript Central® http://mc.manuscriptcentral.com/fems, which is a web-based manuscript submission, reviewing and tracking system used by FEMS. Manuscript Central shortens the time needed for processing and peer reviewing manuscripts and so leads to an improved service to authors.
First-time users should generate a user account. Please also fill in your telephone and fax number in the account fields. Select *FEMS Microbiology Letters* when logging-in.
To submit a manuscript, go to the Submitting Author Center. From there you can click on the **Submit a Manuscript** link in the Menu Bar, which takes you to step one of the seven-step process of submitting a manuscript. The progress bar on the left side of the screen will help you navigate the process.
Fill in the boxes in each step with the appropriate information (required fields (req) are indicated). Use the "Next" button to move from step to step, backtrack to correct errors with the "previous" button or use the progress bar on the left side of the screen. You can copy and paste large amounts of text (such as the title or abstract) to save time.
Authors are encouraged to specify preferred or non-preferred reviewers and preferred Editors for their manuscript. Journal Editors, when assigning reviewers for a manuscript, take preferred and non-preferred reviewers into consideration.
If the submitting (contact) author is not the same as the formal corresponding author, this can be indicated in step five. Please be sure to have this correctly stated on the title page.
After uploading your manuscript files you will get to the last submission step (Review & Submit). Here you must check that the manuscript has been accurately converted to a PDF and HTML file (view pdf or html proof) before clicking the "Submit" **button to complete the submission. If you do not receive email confirmation of your submission within 24 hours contact** submission.letters@fems-microbiology.org. The email confirmation will include a manuscript number, which you should refer to in all correspondence.
More instructions on the submission procedure can be found in the "FEMS Submission Instructions", which can be reached via the "Instructions and Forms" button at the top right of all Manuscript Central pages.
Members of the Editorial board and other appropriate experts will referee the papers. Editors handling papers will independently make decisions on acceptance, revision or rejection based on the referees' reports.

PREPARATION OF MANUSCRIPTS

A recent issue of *FEMS Microbiology Letters* should be consulted for format and style. Please download a sample copy from http://www.elsevier.com/locate/femslett/. Up-to-date Notes to Authors are also available on the Journals page of the FEMS Website (http://www.fems-microbiology.org/).

1. Language

Papers should be in English (consistent either British or American spelling). Authors who are unsure of correct English usage should have their manuscripts checked by someone proficient in the language; manuscripts that are deficient in this respect may be returned to the author for revision before peer review.
English language help service: Upon request, Elsevier will direct authors to an agent who can check and improve the English of their paper (*before submission*). Please contact authorsupport@elsevier.com for futher information.

2. Presentation of manuscripts; preferred format

FEMS Microbiology Letters strongly recommends that you compile your manuscript in MS Word and save it as a DOC file, using the following layout.

a) Start with the title page, followed by the abstract, main text and references.
b) Next add the tables within the same document, starting each new table on a separate page.
c) Follow the tables with the figure legends.
d) Finally add the figures, putting each figure on a separate page ensuring that the figure is at least the size it will be in the final printed document (try not to resize). Add the figure number (e.g. Fig. 1) to each page well outside the boundary of the space occupied by the figure. Check that the incorporated figures can be printed as close as possible to the original resolution of the figures in the package in which they were generated. If you wish to upload separate figure files, Manuscript Central will combine your manuscript main body and figure files into one PDF file. Please ensure that you upload the figures only once, i.e. either embedded at the end of the text document or as separate files.

e) It is essential that both page and line numbering are active in the submitted document.
f) The right-hand margin justification should be switched off. Artificial word breaks at the end of lines must be avoided. The manuscript should be double-spaced (including references, tables and legends for figures) with wide margins. The beginning of each new paragraph must be clearly indicated by indentation.
g) If you do not use MS Word then save in MS Word format in the word processor that you use. Rich text (.rtf) format may also be used.
h) Please, use standard fonts (Arial, Times New Roman, Symbol, Helvetica, Times) otherwise symbols might alter unpredictably. In your Word document, on the Tools menu, click Options, select the Embed TrueType fonts check box and then click the Save tab. Manuscripts must not be submitted with fonts that are not in standard Microsoft UK/US use (fonts that are part of other packages (e.g. Japanese, Chinese or Korean language packages) may block PDF conversion.

3. Length

Priority will be given to short papers. The majority of papers will occupy four to six pages upon publication in the Journal. The text (including abstract but excluding title page, references and legends to figures) should not exceed 3000 words (or about 10 pages of a double-spaced manuscript). References should be kept to a minimum. A combined total of six figures and tables is permitted. If the paper exceeds these guidelines it will be returned for condensation without peer review, unless the authors have provided compelling reasons for the exceptional length.

4. Revision

Manuscripts may be returned to authors for modification of the scientific content and/or for shortening and language correction. Revised versions must be submitted online through Manuscript Central. In "manuscripts with decisions", click on the "create a revision link" that you will find alongside the previous version. At this stage we require a source file of your text and tables (DOC or RTF format, but not PDF). You must clearly indicate the changes that have been made. Figures should be uploaded in separate files and at sufficient resolution (see section 8). All files of the previous version should be deleted from the revised manuscript.

If a paper that is returned to the authors for amendment is not resubmitted in revised form within one month it will be regarded as withdrawn. Any revised version received subsequently will be treated as a newly submitted manuscript and the date of receipt will be altered accordingly. Authors who feel that there are substantial grounds for disagreement with an Editor's decision should contact the Chief Editor, whose decision will be final. Authors who wish to withdraw their manuscript (at any stage of process) sould contact the Editor.

5. Title, authors and keywords

The paper should not form part of a numbered series but should be headed by a concise, informative title. Authors are reminded that titles are widely used in information-retrieval systems.

The title should be followed by the name(s) of the author(s) (with first or middle names in full and including all initials) and by the name(s) and address(es) of the institute(s) where they worked during the study. For multiple authors with different affiliations, please indicate the relevant affiliations. The name and full postal address, telephone and fax numbers, as well as the email address of one corresponding author should be provided in a footnote.

A list of three to six keywords must be included on the first page. Authors are requested to consult the subject indices of *FEMS Microbiology Letters* or the list of subject headings from *Index Medicus* for preferred synonyms and standard abbreviations. Plural terms should be avoided. The title is not used for preparing the index, so important words and phrases may appear in both the title and keywords. General terms, such as 'Enzyme', 'Membrane', 'Transport', etc., should not be used unless qualified, e.g. 'Enzyme activation', 'Membrane phosphorylation', 'Ion transport'.

6. General arrangement of papers

Papers should be written mainly in the past tense, but the present tense can be used to convey authors' ideas and generally accepted information. Hence, *Materials and methods* and *Results* are normally written in the past tense and the present tense is occasionally used in the *Introduction* and *Discussion*.

a) *Abstract*. Limit the abstract to one paragraph not exceeding about 100 words. Some abstracting services use authors' summaries without modification so this section must be intelligible without reference to the full paper. References must not be cited in the abstract and abbreviations should be eliminated.

b) *Abbreviations* should be avoided, but if they have to be used, they must be defined the first time they are used in the main text. Do not abbreviate genus in the title, keywords, or at first use in the abstract and introduction.
c) *Introduction.* This should state the objectives but should not contain a summary of the results. Sufficient information should be given to explain the background to non-specialists but there is usually no need for a comprehensive literature survey.
d) *Materials and methods.* Sufficient detail must be provided to allow the work to be independently repeated and verified. Suppliers and manufacturers of materials and their brief address should be included.
e) *Results* (the presentation of data is given below).
f) *Discussion.* This should not simply recapitulate the Results. A combined results and discussion section is also encouraged.
g) *Acknowledgements* can be made to funding agencies, colleagues and members of a laboratory who provided unpublished data or assisted with the review, etc.
h) *References* (the style and format are given below).

7. Preparation of supplementary data

Elsevier now accepts electronic supplementary material to support and enhance your scientific paper. Supplementary files offer the author additional possibilities to publish supporting applications, movies, animation sequences, high-resolution images, background datasets, sound clips and more. Supplementary files will be published online alongside the electronic version of your article in Elsevier web products, including ScienceDirect: http://www.sciencedirect.com. To ensure that your submitted material is directly usable, please provide it in one of Elsevier's recommended file formats (http://authors.elsevier.com). Authors should submit the material in electronic format together with the article. Select ''Supporting Documentation'' or ''MultiMedia'' for your file designation when uploading the file through the online submission system. Upload a separate DOC file listing concise and descriptive captions for each file uploaded as supplementary data. Please also indicate that you have uploaded these files in your cover letter. For detailed instructions please visit Elsevier's Author Gateway at http://authors.elsevier.com.

8. Presentation of data

Do not tabulate or illustrate points that can be adequately and concisely described in the text. Do not repeat information in both tables and figures. Figures and tables, along with their legend (and/or footnote), should be understandable in their own right without having to refer to the main text.

(a) *Tables.* Tables and legends should be typed double-spaced. Explanatory footnotes should be related to the legend or table using superscript, lower-case letters. All abbreviations should be defined after the footnotes below the table or by reference to a previous table in the same paper.

Table 1
Growth rates of *R. palustris* strains on BA, 3-, 2-, and 4-CBA as the sole carbon source under anoxic conditions in the light

Strain[a]	Maximum specific growth rate (h^{-1}) on[b]:			
	Parental strain	Variant		
	BA	3-CBA	2-CBA	4-CBA
API	0.060	0.029	0.005	0.003
KD1	0.074	0.019	(0.002)[c]	NG[d]
BIS10	0.039	0.020	0.005	0.002
WS17	0.062	0.020	0.008	0.002
NCIB8288	0.073	0.018	0.011	0.003
NCIMB8252	0.032	0.006	0.005	NG

[a] All parental strains were only able to degrade 3- and 2-CBA when BA (1 mM) was present as a co-substrate.
[b] BA was used at 2 mM final concentration and 3-, 2-, and 4-CBA were used at 1 mM final concentration. The data shown are mean values of duplicate measurements.
[c] Growth started only after 5 weeks of incubation.
[d] NG, no growth ($OD_{660\ nm} < 0.1$).

(b) *Line art is an artwork type commonly used for graphs and charts containing scientific data as well as hand-drawn images.* Figures should be designed for maximum clarity, to economise on space and to take account of the dimensions of the printed column or page. Columns are 8.5 cm wide and pages are 17.8 cm wide. Line art must be drawn following these specifications:

- Figures should be 17 cm in width (twice their final size) with wide margins.
- All lines should be drawn at 1.5 point (0.5 mm wide), broken line styles may be used to differentiate multiple plot lines if desired.
- Letters and numbers should be 16 point (capitals 4 mm high) non-serif.
- Symbols should be 3 mm in diameter; the following are preferred: ○, ●, □, ■, △, ▲, ▽, ▼. Lines drawn to accompany the points should not go through hollow symbols.
- Grid lines should not be used.
- Numbers used as axis labels should have minimum significant figures; amounts less than unity must carry a preceding zero (e.g. 0.5 not .5).
- The use of concise explanatory labels (e.g. black square, white circle, grey line) aids interpretation and so is encouraged instead of incorporating symbols in the typed legend printed below the figure or as a separate drawn legend within the figure.
- Larger composite figures may be designed to occupy 2 columns when this can achieve an overall saving in space. The character, line and symbol sizes should be adjusted accordingly to achieve the same sizes on the printed page. For example, with a 24 cm-wide figure, the sizes would be: characters, 12 point, capitals, 3 mm high; lines, 1 point, 0.35 mm; symbols, 2.3 mm in diameter.

(c) *Half-tone and colour figures.* Half-tone illustrations should be very sharp with maximum contrast. Magnification should be indicated where appropriate by inclusion of a bar marker. Non-essential areas of photographs should be cut off. Photographs of electropherograms, etc., in which there is poor contrast, may be better replaced by line drawings, but in this case the photographs should be submitted for scrutiny by the Editor. If photographs have been digitally processed to enhance their quality, this should be stated. **Colour illustrations will be published free of charge provided colour is essential for their understanding**. Authors of MiniReviews are encouraged to make optimal use of the free-colour feature. If, together with your accepted article, you submit usable colour figures then Elsevier will ensure, at no additional charge, that these figures will appear in colour on the web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in colour in the printed version. For colour reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article. For further information on the preparation of electronic artwork, please see http://authors.elsevier.com/artwork. Contributions of colour pictures (and black and white figures of course) serving as cover illustrations for the journal are also welcome.

(d) *Electronic submission of Figures.* High-quality figures are required when the final version of the manuscript is submitted through Manuscript Central, and should be prepared using the following guidelines.

- Please use quality graphic programs such as Adobe Photoshop or Adobe Illustrator. When using other applications, such as Corel Draw or Freehand, please export to TIFF (see also http://authors.elsevier.com/artwork, application guideline box, bottom left corner).
- Please make sure your electronic figure is at the desired size for the printed article.
- For halftones you need a minimum of 300 dpi; combination artwork a minimum of 500 dpi; and for line figures preferably 1000 dpi to 1200 dpi.
- Combination artwork (artwork containing half-tone and line art elements, e.g. electrophoresis gels or Southern blots with lane and fragment sizes labeled) must be in EPS. In case the combination artwork is scanned the preferred format is TIFF with a resolution of minimal 500 dpi using LZW compression.
- The following figure formats are acceptable for our publisher (provided they are created with the instructions given above):
  - TIFF and EPS (please make sure to embed all used fonts)
  - Word (DOC), Excel (XLS) or PowerPoint (PPT)
  - Illustrator (AI) or Photoshop files (PSD)
- For each figure one file must be submitted.

You will find helpful and simplified guidelines by clicking the "FEMS Revision Instructions, including figures" icon, which can be reached via the "Instructions and Forms" button at the top right of all Manuscript Central pages. Detailed information can be found at http://authors.elsevier.com/artwork.

In principle the electronic files will be used for producing the final publication, but the Publisher may request a set of high-quality printouts of your figures for production purposes.

(e) *Figure legends.* Legends for both line-drawings and half-tones must be given on a separate page in the main text. The legends should consist of a preliminary sentence constituting a title, followed by a brief description of the way the particular experiment was carried out (but not repeating the Materials and methods section), and any other necessary description of symbols or lines. All abbreviations must be defined.

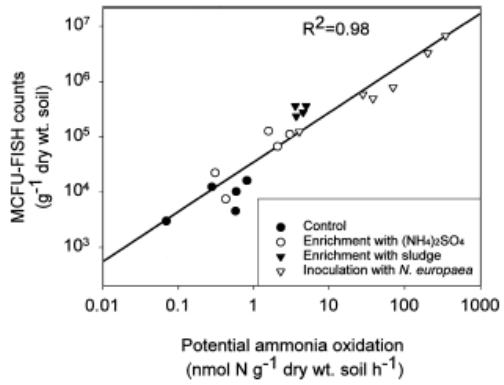

Fig. 4. Correlation between potential ammonia oxidation and MCFU-FISH counts of AOB in the bacterial fractions extracted after various soil treatments. Each point represents mean values for MCFU-FISH and potential ammonia oxidation after the same treatment and incubation time.

9. Reproducibility of results and statistical tests

Authors should state how many times experiments were repeated and whether mean or representative results are shown. Variability should be indicated statistically wherever possible as part of, but not in place of, a proper statistical analysis. If results are expressed as percentages, the absolute value corresponding to 100% must be stated. Avoid values with unjustified numbers of significant figures; in most cases three significant figures is consistent with the accuracy attained in microbiological experiments.

Results of statistical tests should be presented wherever possible as evidence for conclusions. Such information must be presented concisely to illuminate the results, but not to dominate them. The tests used should be briefly described in the Materials and methods section. Details of the diagnostic checks made for the assumptions of the statistical tests and for the validity of any transformations used should be stated clearly. Further information can be found in the following references: (a) Sokal, R.R. and Rohlf, F.J. (1981). Biometry. W.H. Freeman, San Francisco; (b) Fry, J.C. (1993). Biological Data Analysis: A Practical Approach. IRL Press, Oxford.

10. Nomenclature, abbreviations and units

Authors should follow internationally accepted rules and conventions. Authors should provide evidence for thorough identification of new isolates and use the most recent acceptable name.

Microorganisms. The spelling of bacterial names should follow the Approved Lists of Bacterial Names http://www.bacterio.cict.fr/. If there is a reason to use a name that does not have standing in nomenclature, the name should be enclosed in quotation marks and an appropriate statement concerning the nomenclatural status of the name should be made in the text (for an example, see Int. J. Syst. Bacteriol. (1980) 30, 547–556).

Viruses. Names used for viruses should be those approved by the International Committee on Taxonomy of Viruses (ICTV) http://www.ncbi.nlm.nih.gov/ICTV/. If desired, synonyms may be added parenthetically when the name is first mentioned. Approved generic (or group) and family names may also be used. Please note that *FEMS Microbiology Letters* will publish research papers on bacteriophages, but not on viruses.

Enzymes. For enzymes, use the recommended (trivial) name assigned by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology as described in Enzyme Nomenclature http://www.chem.qmw.ac.uk/iubmb/enzyme/.

Genes. Genetic nomenclature should essentially follow the recommendations of Demerec et al. (Genetics (1966) 54, 61–76), and those given in the instructions to authors of the *Journal of Bacteriology* and *Molecular and Cellular Biology* (January issues).

Biochemical compounds. Consult the European Journal of Biochemistry or the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (http://www.chem.qmw.ac.uk/iubmb/).

Abbreviations. Abbreviations should only be used as an aid to the general reader and their use should be strictly limited. Define each abbreviation and introduce it in parentheses the first time it is used: e.g. 'cultures were grown in Eagle minimal essential medium (MEM)'. Eliminate abbreviations that are not used at least six times in the manuscript.

In addition to abbreviations to the international system of units of measurements, other common units (e.g., bp, kb, Da), chemical symbols for the elements, and the standard biochemical abbreviations (see Eur. J. Biochem.) should be used without definition.

It is often possible to use pronouns or to paraphrase a long word after its first use (e.g. 'the drug', 'the substrate'). Standard chemical symbols and trivial names or their symbols (folate, Ala, Leu, etc.) may be used for terms that appear in full in the neighbouring text.

Abbreviations other than those recommended by the IUPAC-IUB (Biochemical Nomenclature and related Documents, 1978) should be used only when a case can be made for necessity, such as in tables and figures.

Reporting numerical data. The international system of units (SI) should be used; ml is acceptable in place of cm^3 for liquid measures. The form for units is $\mu g\ ml^{-1}$ and not μg/ml; parentheses should be used to improve clarity, e.g. ml (g dry wt soil)$^{-1}$ h^{-1}. Multiplication of numbers should be indicated by a multiplication sign with spaces either side (e.g. 6.2×10^8) and of units by a space (e.g. mg l^{-1}).

The prefixes k, m, μ, n, and p should be used in combination with the standard units for reporting length, weight, volume and molarity for 10^{-3}, 10^{-6}, 10^{-9}, and 10^{-12}, respectively. Use $\mu g\ ml^{-1}$ or $\mu g\ g^{-1}$ instead of the ambiguous ppm. Units of temperature are presented as follows: 37 °C or 324 K.

11. References

These should be numbered sequentially in the order of their citation in the text and inserted between square brackets, e.g. [1], [16–21]. The list of references should follow the order of their citation and should be double-spaced. References to journals should contain the names and initials of all authors, year of publication in parentheses, title of the paper, and the abbreviation of the title of the periodical according to *Index Medicus*. These should be followed by the volume number and first and last page numbers. References should not be given to work 'in press' unless it has been accepted for publication and if so, then journal name and year of publication should be provided. References to books should include the title of the book, the year of publication, the publishing company and the place of publication.

Examples:

[1] O'Donnell, C.M. and Edwards, C. (1992) Nitrosating activity in *Escherichia coli*. FEMS Microbiol. Lett. 95, 87–94.
[2] Dinter, Z. and van Morein, B. (1990) Virus Infections in Ruminants, 592 pp. Elsevier, Amsterdam.
[3] McCarthy, A.J. (1989) Thermomonospora. In: Bergey's Manual of Systematic Bacteriology (Williams, S.T., Sharpe, M.E. and Holt, J.G., Eds.), Vol. 4, pp. 2552–2572. Williams and Wilkins, Baltimore, MD.

Unpublished results and personal communications may be mentioned in the main text provided that (a) the names and initials of all the persons involved are listed, and (b) they have all granted permission for the citation. Unpublished accession numbers for nucleotide sequences and similar information must be accompanied by sufficient details to allow the relevant information to be retrieved.

12. Nucleotide and amino acid sequences

Any newly determined nucleotide and amino acid sequences must be deposited in an appropriate data bank. Authors are encouraged to use the EMBL Data Library but can also use other archives, such as GenBank. **An accession number must be obtained before submission to the Editor and this fact should be mentioned in the cover letter**. The accession number should be included in the main body at the end of the Materials and methods section. Authors should also include the accession number in the appropriate figure legend or refer to the Materials and methods section. The sequence data should be released to the public no later than the online publication date. Authors wishing to enable other scientists to use the accession numbers cited in their papers via links to these sources should type this information in the following manner:

For each and every accession number cited in an article, authors should type the accession number in **bold, underlined text**. Letters in the accession number should always be capitalised (see example). This combination of letters and format will enable Elsevier's typesetters to recognise the relevant texts as accession numbers and add the required link. Example: "GenBank accession nos. **AI631510**, **AI631511**, **AI632198**, and **BF223228**), a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. **BE675048**), and a T-cell lymphoma (GenBank Accession No. **AA361117**)".

Authors are advised to check accession numbers very carefully. **An error in a letter or number can result in a dead link**. In the final version of the **printed** article, the accession number text will not appear bold or underlined. In the final version of the **electronic copy**, the accession number text will be linked to the appropriate source in the NCBI databases enabling readers to go directly to that source from the article.

Nucleic acid and amino acid sequences submitted as figures should be as sharp and clear as possible and retain perfect legibility after reproduction. There should be no confusion between G and C. The width should be (close to) that of the typesetting area (i.e., 178 mm) or column width (85 mm). Any additional markings should be clearly added in black ink or with a computer drawing package.

13. Careful checking
Manuscripts should be checked carefully before submission since substantial alterations made at the 'proof' stage may be charged for by the Publisher.

14. Enquiries and alterations
Queries will be referred back by email to the corresponding author. The Editors reserve the right to make minor alterations to the text without altering the scientific content.

PROOFS

A manuscript received by the Publisher is considered to be in its final form. One set of page proofs in PDF format will be sent by e-mail to the corresponding author, to be checked for typesetting/editing. A form with queries from the copyeditor may accompany the proof. It is important to ensure that all corrections are sent back to Elsevier in one communication, as subsequent corrections will not be possible. The Publisher reserves the right to proceed with publication if corrections are not communicated within 24 hours.

OFFPRINTS

The corresponding author will receive, upon acceptance of an article by the Publisher, a Copyright Transfer form (or Licence Agreement) and an Offprint Order form. On the Offprint Order form one can indicate the address to which the free offprints are to be sent and additional offprints may be ordered. **Twenty-five free** offprints are available for each paper.

NO PAGE CHARGES

There is no page charge for publication in any of the FEMS publications.

ETHICAL AND RELATED ASPECTS

The Editors expect that new and variant organisms, viruses and vectors described in FEMS journals will be made available, under written request and for their own use, to all qualified members of the scientific community. If delays in strain or vector distribution are anticipated or if they are available from sources other than the authors, this should be indicated. The Editors encourage authors to deposit important strains in publicly accessible culture collections and to refer to the collections and strain numbers in the text. In the case of materials that have been distributed by individuals, authors should indicate the laboratory strain designations and name and address of the donor as well as the original culture collection identification number, if any.
Papers describing experimental work with humans must include a statement that the Ethical Committee of the institution in which the work was done has approved it, and that the subjects gave informed consent to the work. Experiments with animals or with genetically manipulated organisms must have been done in accordance with the legal requirements of the relevant local or national authority. Procedures should be such that experimental animals do not suffer unnecessarily.
Submission of a manuscript implies that the work described has not been published before (except in the form of an abstract or as part of a published lecture, review or academic thesis, in which case reference should be made in a footnote to the title) and that it is not under consideration for publication elsewhere. **The corresponding author must ensure that its publication has been approved by all co-authors and tacitly or explicitly by the responsible authorities in the laboratories where the work was carried out and that all persons entitled to authorship have been named.** If accepted, the article must not be published elsewhere in the same form in either the same or another language, without the consent of the Editors and Publisher. Each named author must be responsible for at least the part describing his or her contribution and must have seen the entire final text before submission and any substantial subsequent revisions. The Editors must be notified in writing by the corresponding author of any deviation from these rules. The articles published in *FEMS Microbiology Letters* represent the scientific findings and opinions of the authors. Whilst the Editors and Publisher make every effort to ensure the accuracy of all published matter, they can accept no responsibility or

liability, collectively or individually, for any erroneous, misleading or unintentionally damaging statements, which may appear in the journal. Authors must draw attention to chemical or biological hazards that may be involved in materials and methods used in experiments.

REQUESTS FOR PERMISSION TO REPRODUCE MATERIAL FROM PUBLISHED ARTICLES

Individuals wishing to reproduce figures, tables and excerpts of text (not exceeding 250 words) from articles published in *FEMS Microbiology Letters* for non-commercial purposes may do so providing the original publication is acknowledged accordingly and the authors' approval is obtained, and in this case no special permission is needed from the publisher. Authors may also include the article in a thesis without special permission. In all other cases, permissions may be sought directly from Elsevier's Rights Department in Oxford, UK: phone (+44) 1865 843830, fax (+44) 1865 853333, e-mail permissions@elsevier.com. Requests may also be completed on-line via the Elsevier homepage (http://www.elsevier.com/locate/permissions).

INFORMATION FOR AUTHORS AND READERS

The Editors and publishers are always willing to hear from authors and readers and consider their views sympathetically. Please note the following contact details:

- For helpful and simplified guidelines on the submission of high-quality figures for the final version of your manuscript, please refer to "FEMS Revision Instructions, including figures" icon, which can always be reached via the "Instructions and Forms" button at the top right of all Manuscript Central pages. Further detailed information on figures can be found at http://authors.elsevier.com/artwork
- Queries related to submission: email submission.letters@fems-microbiology.org
- Manuscripts under review: consult the Submitting Author Center at http://mc.manuscriptcentral.com/femsle
- Status of accepted articles: consult the Author Gateway from Elsevier at http://authors.elsevier.com
- Questions arising after acceptance of an article: email authorsupport@elsevier.com