UNIVERSIDADE FEDERAL DE PERNAMBUCO
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DEPARTAMENTO DE MICOLOGIA
PÓS-GRADUAÇÃO EM BIOLOGIA DE FUNGOS

POTENCIAL BIOTECNOLÓGICO DE *CANDIDA LIPOLYTICA* NA PRODUÇÃO DE BIOSSURFACTANTES, NOS PROCESSOS DE REMOÇÃO E BIOSSORÇÃO DO PIRENO (DERIVADO DO PETRÓLEO)

MABEL HANNA VANCE-HARROP

Recife

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Tese apresentada ao Curso de PósGraduação em Biologia de Fungos da Universidade Federal de Pernambuco,como parte dos requisitos, para obtenção do título de Doutor em Biologia de Fungos.
Área de Concentração: Micologia aplicada.

Autora: MABEL HANNA VANCE-HARROP

Orientadora: Profa. Dra. GALBA MARIA DE CAMPOS-TAKAKI

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Mabel Hanna Vance-Harrop

Comissão Examinadora

Profa. Dra. Galba Maria de Campos-Takaki (Orientadora)

Departamento de Química - NPCIAMB/UNICAP, Recife, PE

Profa. Dra.Norma Buarque de Gusmão

Departamento de Antibióticos - UFPE, Recife, PE

Profa. Dra. Maria Aparecida de Resende

Departamento de Microbiologia, ICB - UFMG, Belo Horizonte, MG

Profa. Dra. Ana Lúcia Figueiredo Porto

Departamento de Morfologia e Fisiologia Animal - UFRPE, Recife, PE

Profa. Dra. Kaoru Okada

Departamento de Biologia - NPCIAMB/UNICAP, Recife, PE

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ÍNDICE

LISTA DE FIGURAS

REVISÃO DA LITERATURA

CAPÍTULO I

CAPÍTULO II

CAPÍTULO III

CAPÍTULO IV

LISTA DE TABELAS

REVISÃO DA LITERATURA

CAPÍTULO II

CAPÍTULO III

RESUMO

Em prévios estudos, *Candida lipolytica* IA 1055,demonstrou excelente potencial na produção de biossurfactantes com habilidade de emulsificação, em meios de cultura de baixo custo. Considerando o potencial biotecnológico de *C. lipolytica*, novas investigações foram realizadas utilizando os meios de cultura de baixo custo, a base de água do mar, adicionados de óleo de babaçu e glicose como controle da fonte de carbono. Observou-se maior produção de biossurfactantes com os meios Yeast Salt Water-Babaçu (YSW-B^2) e Yeast-Salt Water-Babaçu (YSW-B^3) cujas moléculas produzidas apresentaram excelente capacidade de emulsificação, sendo consideradas como novos bioemulsificantes, constituídos quimicamente por carboidratos, proteínas e lipídeos. A partir dos meios selecionados foi realizado um planejamento fatorial de dois níveis com cinco fatores (extrato de levedura, sulfato de amônio, uréia, fosfato de potássio e óleo de babaçu), com a finalidade de testar a sua influência sobre a produção de biossurfactante, através dos efeitos estatisticamente combinados. Os resultados obtidos sugeriram maior produção com os níveis utilizados do extrato de levedura, fosfato e da uréia nos seus valores superiores, enquanto que o da amônia, foi fixado em seu valor inferior. Quanto a fonte de carbono, o óleo de babaçu, os níveis fracionários não indicaram um efeito significativo sobre a resposta, ou seja, o aumento da produção de biossurfactante. As condições dos meios de cultura selecionados pelo planejamento fatorial foram utilizadas para a realização de estudos com substratos mistos (solúvel e insolúvel) para os processos de remoção e biossorção do pireno (derivado do petróleo) em cinco diferentes meios de cultura. Os resultados das análises por Cromatografia Líquida de Alta Eficiência (CLAE), demonstraram uma alta taxa de remoção do pireno nos meios I e IV, com percentuais de 99,02% e 97,51%, respectivamente. Estudos subseqüentes, com a biossorção do pireno foram realizados utilizando a biomassa de *C. lipolytica* imobilizada em álcool polivinil, como matriz inerte. Várias concentrações de biomassa (100, 200 e 300 mg) foram imobilizadas e empacotadas em colunas para avaliação do processo de biossorção do pireno. Os eluentes (água e acetato de etila) e as frações obtidas foram analisados por CLAE. Os resultados obtidos sugerem que a biomassa imobilizada de *C. lipolytica* apresenta grande potencial nos processos de biossorção do pireno tendo em vista o baixo custo, da produção e da imobilização da biomassa. Estes resultados indicam que a capacidade máxima de biossorção do pireno foi de 86,91; 44,62 e 29,43 mg g^{-1} , respectivamente para as colunas com 100 mg, 200 mg e 300 mg de biomassa.

ABSTRACT

In previous studies *Candida lipolytica* IA1055 has shown excellent potential in biosurfactant production, demonstrating emulsifying ability in low-cost culture media. Taking that into consideration, investigations were performed utilizing low-cost culture media in basal seawater supplemented with babassu oil and glucose as control of carbon sources. The highest biosurfactant production was observed in media Yeast Salt Water – Babassu (YSW-B^2) and Yeast Salt Water – Babassu (YSW-B^3), in which the molecules produced showed excellent emulsification activity. These molecules were considered new bioemulsifiers, chemically consisting of carbohydrate, protein and lipid. After media selection, a two-level factorial design with five factors (yeast extract, ammonium sulfate, urea, potassium phosphate and babassu oil) was performed, in order to test their influence over biosurfactant production, through statistically combined effects. The results suggested that higher production occurred with yeast extract, urea and potassium phosphate in their highest levels, and ammonium in its lowest level. As for the carbon source (babassu oil), the results indicated that its level was independent to the biosurfactant production. The culture media conditions, selected by the factorial design, were used to perform studies with mixed substrates (soluble and insoluble) for the processes of removal and biosorption of pyrene (petroleum derivative) using five different culture media. The results of High Performance Liquid Chromatography (HPLC) analyses demonstrated a high removal rate of pyrene in media I and IV with 99.02% and 97.51% percentage, respectively. Subsequential studies were performed with the biosorption of pyrene utilizing *Candida lipolytica* immobilized biomass in polyvynil alcohol as inert matrix. Biomass concentrations (100, 200 and 300 mg) were immobilized and packed in columns for evaluation of pyrene biosorption process. The eluents (ethyl acetate and water) and the fractions obtained were analyzed by HPLC.The results obtained suggested that the *Candida lipolytica* immobilized biomass presented great potential in pyrene biosorption processes because of its low cost. The results indicated that the maximum biosorption capacity of pyrene was 86.91 , 44.62 and 29.43 mg l^{-1} respectively for the 100 mg, 200 mg and 300 mg colunms.

INTRODUÇÃO GERAL

Nas últimas décadas houve um aumento da população, gerando uma preocupação em torno dos potenciais efeitos adversos para a saúde do homem e também para a ecologia da produção, uso e distribuição de numerosas substâncias químicas resultantes do desenvolvimento industrial, das atividades na agricultura e dos resíduos gerados pelas atividades urbanas e domésticas. Portanto, a qualidade de vida está ligada intrinsecamente à qualidade total do meio ambiente em resposta ao crescimento populacional e ao aumento na demanda por reservas naturais de ar, água e solo, devendo-se uma atenção global para a descoberta de novas maneiras de sustentar e cuidar do meio ambiente (KOLPIN *et al.*, 2002).

Assim, devido ao aumento pronunciado da exploração de petróleo e outras fontes de energia relacionadas, a atenção pública tem sido direcionada para os efeitos ambientais dessa exploração, particularmente, para o potencial de contaminação do meio ambiente pelo petróleo e seus subprodutos (WALKER; COLWELL, 1976).

O petróleo é uma mistura viscosa líquida que contém milhares de componentes formados principalmente, por carbono e hidrogênio. Todos os subprodutos do petróleo são derivados do óleo cru, cujos principais constituintes são os hidrocarbonetos (ATLAS, 1981).

Os produtos derivados do petróleo são a principal fonte de energia para a indústria e a vida cotidiana. O petróleo é também a matéria-prima para muitos produtos químicos, como plásticos, tintas e cosméticos. O transporte de petróleo através do mundo é freqüente e a quantidade de estoques da substância nos países desenvolvidos é enorme, o que aumenta a possibilidade de acidentes como o derramamento (HARAYAMA *et al.*, 1999).

Uma vez que a primeira linha de defesa, por assim dizer, contra a poluição ambiental por óleos é a população microbiana, torna-se imperativo o conhecimento sobre esses microrganismos

degradadores, presentes na água e no solo (WALKER; COLWELL,1976). Uma estratégia biológica que pode facilitar o contato entre os microrganismos e os hidrocarbonetos insolúveis em água é a emulsificação dos hidrocarbonetos que induzem ao crescimento de microrganismos através da produção de surfactantes. Estes surfactantes auxiliam na dispersão do óleo, aumentam a área superficial para o crescimento e ajudam na separação do microrganismo das gotículas de óleo após o hidrocarboneto utilizável ter sido consumido. A ação dos biossurfactantes na biorremediação do petróleo geralmente ocorre no mínimo por dois processos; o aumento da área superficial dos substratos hidrofóbicos e o aumento da bio-acessibilidade destes compostos (RON, ROSENBERG, 2001).

No Brasil, resultados de trabalhos realizados principalmente por Wilcke *et al*. (2003) demonstraram a presença de hidrocarbonetos aromáticos policíclicos em inúmeras fontes como solo, madeira e ninhos de cupim em diferentes regiões ecológicas do país.

A levedura Ascomycetes *Candida lipolytica* (*Yarrowia lipolytica*), utilizada para estudos de pesquisas fundamental e biotecnológica sobre a produção de biomoléculas, possui habilidade para degradar substratos insolúveis como fonte de carbono, entre os quais o querosene e a gasolina, em meios de cultura contendo componentes de petróleo como única fonte de energia (OKPOKWASILI; AMSCHUKWU, 1988; BARTH; GAILLARDIN, 1997).

Tendo em vista o potencial biotecnológico demonstrado por *Candida lipolytica* (UCP 00065), os objetivos deste trabalho foram desenvolvidos em etapas:

- Verificar a influência dos surfactantes produzidos por *Candida lipolytica* em meios de baixo custo na biodegradação de hidrocarbonetos aromáticos policíclicos;
- Produzir novos bioemulsificantes por *Candida lipolytica* utilizando D-glicose e óleo de babaçu como fonte de carbono;

- Produzir surfactantes por *Candida lipolytica* em meios semidefinidos utilizando planejamento fracionário;
- Utilizar condições de co-metabolismo para a remoção do pireno por *Candida lipolytica*;
- Avaliar a adsorção de pireno por biomassa de *Candida lipolytica* imobilizada.

REVISÃO DA LITERATURA

1. Biossurfactantes

Em sistemas heterogêneos, os limites são de fundamental importância para o comportamento do sistema como um todo. Os biossurfactantes são substâncias que adsorvem e alteram as condições que prevalecem nas interfaces. São moléculas anfipáticas, compostas por uma porção hidrofílica e uma porção hidrofóbica, com capacidade para reduzir a tensão superficial e interfacial entre interfaces líquidas, sólidas e gasosas, permitindo a essas se misturarem ou dispersarem como emulsões em água ou outros líquidos (COOPER,1986).

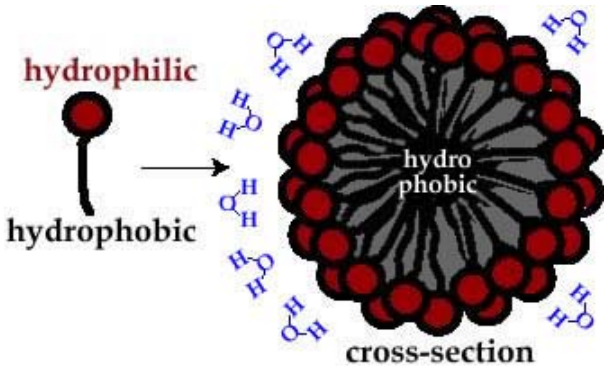


Figura 1. Molécula de um surfactante.

Fonte: http://www.virtuallaboratory.net

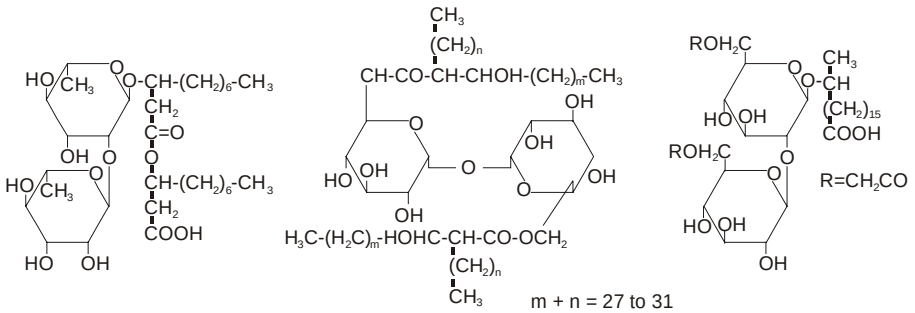
1. 1. Estrutura e origem microbiológica

Os biossurfactantes são classificados principalmente por sua composição química. Suas estruturas incluem uma porção hidrofílica, que consiste de aminoácidos ou peptídeos, mono, di ou polissacarídeos, e uma porção hidrofóbica de ácidos graxos, saturados ou insaturados. As principais classes de biossurfactantes são glicolipídeos, lipopeptídeos, lipoproteínas, fosfolipídeos, ácidos graxos e biossurfactantes poliméricos. Embora haja um grande número de relatos sobre a síntese de biossurfactantes por microrganismos que degradam hidrocarbonetos (CIRIGLIANO; CARMAN, 1984; NEU *et al*., 1992; BANAT, 1993), há também relatos sobre biossurfactantes produzidos por compostos solúveis em água, como carboidratos (COOPER; PADDOCK, 1984; JOHNSON *et al*., 1992; MOSCOVICI *et al*., 1996). Enfim, ocorre a produção de biossurfactantes com ambos os substratos, solúveis e insolúveis (CASAS; GARCIA-OCHOA, 1999; MACCAFFREY; COOPER, 1995; ZHOU; KOSARIK, 1995). Os microrganismos que produzem biossurfactantes estão distribuídos numa grande variedade de gêneros. Os principais tipos de biossurfactantes e sua origem microbiológica estão demonstrados na Tabela 1. Algumas estruturas típicas dos biossurfactantes podem ser observadas na Figura 2.

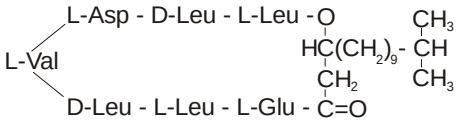
Tabela 1. Principais biossurfactantes e sua origem microbiológica

Biossurfactantes	Organismos
Glicolipídeos	
Rhamnolipídeos	*Pseudomonas aeruginosa*
	Pseudomonas sp.
Trealolipídeos	*Rhodococcus erythropolis*
	Nocardia erythropolis
	Mycobacterium sp.
Lipídeos sulfurosos	*Torulopsis bombicola*
	T. apicola
	T. petrophilum
Celobiolipídeos	*Ustilago zeae*
	U. maydis
Lipopeptídeos e lipoproteínas	
Peptídeo-lipídeo	*Bacillus licheniformis*
Viscosina	*P. fluorescens*
Surfactina	*B. subtilis*
Subtilisina	*B. subtilis*
Gramicidina	*B. brevis*
Polimixina	*B. polymyxa*
Ácidos graxos, lipídeos neutros e fosfolipídeos.	
Ácidos graxos	*Corynebacterium lepus*
Lipídeo neutro	*Nocardia erythropolis*
Fosfolipídeos.	*Thiobacillus thiooxidans*
Biossurfactantes poliméricos	
Emulsan	*Acinetobacter calcoaceticus*
Biodispersan	*A. calcoaceticus*
Proteína-manana-lipídeo	*Candida tropicalis*
Liposan	*C. lipolytica*
Carboidrato-proteína-lipídeo	*P. fluorescens*
	Debaryomyces polymorphis
Proteína PA	*P. aeruginosa*

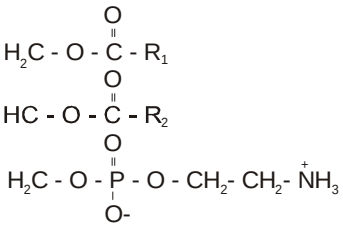
Fonte: Desai; Banat,1997.



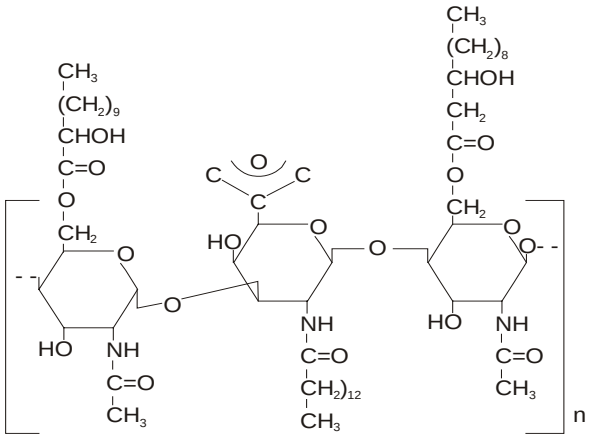
(A) RHAMNOLIPÍDEO (B) TREALOLIPÍDEO (C) LIPÍDEO SULFUROSO



(D) SURFACTINA



(E) FOSFATIDILETANOLAMINA



(F) EMULSAN

Figura 2. Estruturas químicas de biossurfactantes

Fonte: Desai; Banat,1997

1. 2. Propriedades

A propriedade mais importante da molécula do biossurfactante é a de reduzir a tensão superficial e interfacial entre sistemas de polaridades diferentes (COOPER, 1986). Além disso, essa molécula pode atuar como agente dispersante, estabilizante e espumante (GARTI, 1999), como também desestabilizar sistemas estáveis de surfactantes (NADARAJAH *et al*., 2002). Esta molécula apresenta ainda propriedades umidificantes (COOPER; ZAJIC, 1980) e atividades antimicrobianas (KITAMOTO *et al.,* 1993; YAKIMOV, 1995; VOLLENBROICH *et al*., 1997).

Os biossurfactantes apresentam várias vantagens em relação aos surfactantes químicos: baixa toxicidade, alta degradabilidade e melhor compatibilidade ambiental (DESAI; BANAT, 1997).

1. 3. Produção de biossurfactantes

1. 3. 1. Fisiologia e genética

O principal papel fisiológico dos biossurfactantes é dar acessibilidade aos microrganismos, de modo que eles cresçam em substratos insolúveis em água, pela redução da tensão superficial nas interfaces, o que deixa os substratos mais disponíveis para a absorção e o metabolismo. Os mecanismos moleculares envolvidos no processo de absorção desses substratos (ex.: alcanos), contudo, não foram totalmente esclarecidos (FIECHTER, 1992; DESAI; BANAT, 1997).

Os biossurfactantes são produzidos por uma grande variedade de microrganismos, que os secretam, extracelularmente ou ligados a partes das células, geralmente durante o seu crescimento em substratos insolúveis em água (ROSENBERG *et al*., 1979; KOCH *et al.,* 1991). Assim como em outras vias biossintéticas, uma valiosa abordagem no estudo da produção de biossurfactantes tem sido o uso de mutantes (naturais ou induzidos por transposição). Contudo, a escolha desses mutantes é difícil porque a perda da habilidade para produzir o emulsificante geralmente não resulta num fenótipo facilmente selecionável (RON; ROSENBERG, 2001).

A função do biossurfactante na célula microbiana não está esclarecida completamente, contudo, existem especulações acerca do seu envolvimento na emulsificação de substratos insolúveis em água (ZHANG; MILLER, 1995). O contato direto de células com gotas de hidrocarboneto e a interação dessas células com gotas emulsificadas já foram descritas por Rosenberg (1986). Esse mesmo autor, relatou que os biossurfactantes demonstraram envolvimento na aderência celular, promovendo o aumento da estabilidade da célula sob condições ambientais desfavoráveis.

1. 3. 2. Biossíntese

Devido à estrutura anfipática do biossurfactante, duas vias metabólicas primárias, isto é, o hidrocarboneto e o carboidrato, estão envolvidas na síntese de suas porções hidrofóbica e hidrofílica, respectivamente. As vias para a síntese desses dois precursores são diversas e utilizam conjuntos de enzimas específicas. As possibilidades para síntese das diferentes porções dos biossurfactantes e suas reações em cascata são as seguintes:

a) as porções hidrofílica e hidrofóbica são sintetizadas "de novo" por duas vias independentes;

b) a porção hidrofílica é sintetizada "de novo", enquanto a síntese da porção hidrofóbica é induzida pelo substrato;

c) a porção hidrofóbica é sintetizada "de novo", enquanto a síntese da porção hidrofílica é dependente do substrato;

d) a síntese de ambas as porções são dependentes do substrato (SYLDAK; WAGNER, 1987).

1. 3. 3. Regulação

Geralmente, três mecanismos funcionam na regulação da produção de biossurfactantes: a indução, a repressão e os íons nitrogênio e multivalentes (DESAI; BANAT, 1997).

A indução da síntese de trealolipídeos em *Rhodococcus erythropolis* pela adição de hidrocarbonetos foi relatada por Rapp et al. (1979). A indução parece ser também um mecanismo regulador usado para controlar o início da síntese da maioria dos biossurfactantes lipopeptídicos, de acordo com a literatura (KLUGE *et al*., 1988; ULLRICH *et al.,* 1991; BESSON; MICHEL, 1992).

A repressão da produção de biossurfactantes pela bactéria *Acinetobacter calcoaceticus* em substratos insolúveis como hidrocarbonetos foi observada com ácidos orgânicos (GÖBBERT *et al.,* 1984). Com a levedura *Candida lipolytica*, ocorreu a repressão da produção de Liposan quando glicose, acetato e ácidos tricarboxílicos foram adicionados (CIRIGLIANO; CARMAN, 1984). A regulação da síntese de biossurfactantes por fonte de nitrogênio e íons metálicos também merece registro. Guerra-Santos et al. (1984), relataram a influencia da fonte de

nitrogênio (amônia ou nitrato) e extrato de levedura sobre a produção de biossurfactantes. A melhor produção foi obtida com nitrato em comparação com as culturas nas quais a amônia foi utilizada como fonte de nitrogênio. Os resultados descritos por Ristau e Wagner (1983), demonstraram que a deficiência de nitrogênio produziu novos glicolipídeos em fermentação por batelada. Além destes resultados sob a regulação do nitrogênio, uma super produção dos novos glicolipídeos foi obtida pela limitação de íons de metais multivalentes. A síntese de rhamnolipídeos por *Pseudomonas aeruginosa* mostrou efeitos pronunciados pelos íons ferro, magnésio, cálcio, e pelas concentrações do sal de potássio: estes foram investigados com sucesso para o desenvolvimento de um meio de cultura definido (GUERRA-SANTOS et al., 1984; 1986). A limitação do ferro no estímulo à produção de biossurfactantes por *P. fluorescens* foi relatada por Persson et al. (1990), enquanto Cooper et al. (1981) referiram que a adição de sais de ferro e manganês induziram a produção de biossurfactantes por *Bacillus subtilis*.

1. 3. 4. Caracterização genética

Existem relatos sobre o isolamento de mutantes deficientes na produção de biossurfactantes, com a perda concomitante da habilidade para o crescimento em substratos insolúveis em água (SHABTAI; GUTNIK, 1986; RUSANSKY *et al*., 1987). O desenvolvimento recente de técnicas para detectar microrganismos que degradam os hidrocarbonetos acelerou significativamente o isolamento de microrganismos produtores de biossurfactantes. Entre as técnicas para detecção, estão o teste da gota colapso (JAIN *et al.,* 1991), a cromatografia de camada fina (MATSUYAMA *et al*., 1987) e os testes de detecção através de medição da hemólise (MULLIGAN *et al*., 1984).

1. 3. 5. Fatores que afetam a produção de biossurfactantes

Fontes de carbono

As fontes de carbono solúveis em água – como glicerol, glicose, manitol e etanol – foram utilizadas para produção de rhamnolipídeos por *Pseudomonas* sp. Não obstante, a produção de biossurfactante nesse caso foi inferior àquela obtida com substratos insolúveis em água, tais como n-alcanos e óleo de oliva (YAMAGUCHI *et al.,* 1976; ROBERT *et al*., 1989). Foi demonstrado por Edmonds e Cooney (1969) que, na produção de biossurfactante por *Pseudomonas* sp., utilizando-se distintas fontes de carbono com diferentes comprimentos de cadeias no meio de cultura, estas não exibiram efeitos sobre os comprimentos das cadeias de ácidos graxos da porção hidrofóbica da molécula, em glicolipídeos. Por outro lado, Finnerty e Singer (1985) evidenciaram que uma variação qualitativa ocorreu na produção de biossurfactantes por *Acinetobacter* sp. H13-A, refletindo-se sobre o número de carbonos do alcano.

Corynebacterium lepus produziu grande quantidade de biossurfactante extracelular quando cultivado em meio contendo hexadecano como fonte de carbono. O organismo, quando cultivado em glicose, produziu um biosurfactante ligado às células, o qual foi liberado quando tratado com hexadecano após o crescimento (DUVNJAK; KOSARIC, 1985). O trabalho desenvolvido por Banat, (1995) revelou que, quando as células cresceram em uma fonte de carbono solúvel em água (glicose), houve uma baixa produção de surfactante e somente quando toda a fonte de carbono solúvel foi consumida e o meio suplementado com uma fonte de carbono insolúvel em água (ácido oléico), o processo de produção foi iniciado. Glicolipídeos foram produzidos por *Torulopsis bombicola* ATCC 22214 (*Candida bombicola*) ao final da fase

exponencial do crescimento. Dois tipos de fontes de carbono foram utilizados, carboidrato e óleo vegetal, sendo ambos necessários para a obtenção de uma grande produção de surfactantes. A levedura cresceu na fonte de carbono solúvel, e após a fase exponencial, a fonte insolúvel foi adicionada a cultura, ocorrendo um aumento no processo de produção (COOPER; PADDOCK, 1984). Em face de tais evidências, conclui-se que a fonte de carbono disponível, particularmente o carboidrato utilizado, exerce grande influência sobre o tipo de biossurfactante produzido (LEE; KIM, 1993).

Fontes de nitrogênio

Os outros constituintes do meio, além das fontes de carbono, também afetam a produção de biossurfactantes. Entre as fontes de nitrogênio, os sais de amônia mostraram grande influência na produção de biossurfactante pela bactéria Gram-negativa PCB-106 quando utilizados como fonte de nitrogênio (PALEJWALA; DESAI, 1989). A uréia utilizada como fonte de nitrogênio adicional aumentou a produção de biossurfactantes por *Candida bombicola* (RAU *et al*., 1996). O nitrato promoveu a produção máxima por *P. aeruginosa* (GUERRA-SANTOS *et al.,* 1984; MACELWEE *et al*., 1990).

Robert *et al.* (1989) observaram que o nitrato foi a melhor fonte de nitrogênio para produção de biossurfactantes por *Pseudomonas* 44T1, cultivada em óleo de oliva. O efeito da fonte de nitrogênio sobre a produção de rhamnolipídeos por *Pseudomonas aeruginosa* foi observado em meios de cultura contendo proteose peptona e íons amônia e nitrato.A fonte de nitrogênio preferida pelo microrganismo foi o ácido glutâmico da peptona, ocorrendo um aumento simultâneo na produção e na atividade da glutamina sintetase e uma diminuição do crescimento celular (MULLIGAN; GIBBS, 1989). A produção de “lichenysin A”, um

biossurfactante lipopeptídico, foi aumentada em 2 e 4 vezes por *Bacillus licheniformis* BAS50, pela suplementação do meio com ácido glutâmico e L-asparagina, respectivamente (YAKIMOV *et al*., 1996). A limitação do nitrogênio também causou o aumento da produção de biossurfactantes por *Candida tropicalis* II-4 (SINGH *et al*., 1990) e *Nocardia* SFC-D (KOSARIC *et al*., 1990). Guerra-Santos *et al*., (1984; 1986) relataram que as células de *Pseudomonas aeruginosa* (Rsan-ver) alcançaram a produção máxima de rhamnolipídeos utilizando o nitrato como fonte de nitrogênio a uma taxa ótima de C:N de 18. Uma diminuição ou aumento na concentração de nitrogênio foi expressada em uma menor concentração de rhamnose na cultura. De acordo com Hommel *et al*. (1987), o que parece ser importante para a produção de uma biomassa ótima é a quantidade absoluta de nitrogênio, e não a sua concentração relativa. Por outro lado, a concentração da fonte de carbono hidrofílica determina a conversão do carbono disponível para o biossurfactante.

Fatores físico-químicos

Os fatores ambientais e as condições de crescimento, tais como pH, temperatura, agitação e aeração, também afetam a produção de biossurfactantes, através de seus efeitos sobre o crescimento e a atividade das células. O pH do meio desempenhou papel importante na produção de lipídeos sulfurosos por *Torulopsis bombicola* (GÖBBERT *et al*., 1984). *Candida lipolytica* IA 1055 produziu um bioemulsificante extracelular durante o crescimento em glicose, e a máxima produção foi verificada exatamente após o pH alcançar valores aproximados de 3.0 (SARUBBO *et al.,* 2001). Ainda, a atividade de emulsificação máxima produzida por essa levedura em meio de cultura com hexadecano como fonte de carbono foi obtida em valores de pH de 2 a 5 (CIRIGLIANO; CARMAN, 1984). A produção de biossurfactantes por uma amostra termofílica

de *Bacillus subtilis* foi demonstrada em cultivo com sacarose a 2% (m/v) como fonte de carbono, a uma temperatura de 45°C, com concentração de cloreto de sódio de 4% (m/v) na ampla faixa de pH 4,5 a 10,5 (MAKKAR; CAMEOTRA, 1997). Outra amostra de *Bacillus* sp. produziu surfactante sob a mesma temperatura e faixa de pH de 6,5 a 6,8, utilizando glicose e ácido oléico como fontes de carbono (BANAT, 1993). A concentração de cloreto de sódio também pode afetar a produção de biossurfactantes, dependendo do seu efeito sobre a atividade celular. Alguns biossurfactantes foram produzidos em salinidade de cerca de 13%, de água do mar natural sem contudo, serem afetados (VANCE-HARROP *et al*., 2003).

1. 3. 6. Produção de biossurfactantes por biotransformação

Nos últimos anos, uma considerável atenção tem sido voltada à produção de biossurfactantes através da via da biotransformação. Isso se deve principalmente à similaridade estrutural entre os ésteres de sacarose, um grupo de surfactantes comerciais, e os biossurfactantes glicolipídeos (SARNEY; VULFSON, 1995).

O principal impulso nessa direção tem sido o uso de cultura de microrganismos para obter diferentes porções hidrofílicas e hidrofóbicas dos biossurfactantes, as quais podem ser ligadas através de tratamentos enzimáticos para gerar surfactantes comerciais. Esses tratamentos enzimáticos são altamente específicos, e suas reações podem ser realizadas facilmente em condições normais de temperatura e pressão. A mais simples transformação relatada se deu através do uso de amostras de leveduras selecionadas para melhorar a qualidade do óleo pela dessaturação ou saturação dos ácidos graxos componentes do óleo (MONTET *et al.,* 1985). Em um sistema bifásico de D-sorbitol em água e ácido decanóico, a esterificação é catalisada por lipase de *Candida rugosa*. A conversão, a uma taxa de 6.8 $mmol.gl^{-1}.h^{-1}$ durante 24 horas,

ocorreu sem nenhuma redução significativa na taxa de produção alcançada (JANSSEN *et al*., 1990). Sarney e Vulfson (1995) apresentaram detalhes esclarecendo as perspectivas e limitações da síntese enzimática dos surfactantes. Segundo estes autores, o problema fundamental na síntese é a eficiência na solubilização dos constituintes hidrofílicos e lipofílicos na reação da mistura. Este processo oferece algumas vantagens básicas, incluindo uma grande flexibilidade quanto à variação da estrutura química dos produtos.

Isolamento dos biossurfactantes

Os processos de isolamento em muitos procedimentos biotecnológicos são responsáveis por até 60% do custo total da produção. O isolamento dos biossurfactantes depende principalmente da sua troca iônica, solubilidade em água e localização: intracelular, extracelular ou ligado à célula (DESAI; BANAT, 1997).

Entre as técnicas de isolamento de biossurfactantes mais comumente utilizadas estão as extrações com solventes orgânicos como: clorofórmio, metanol, diclorometano, entre outros. Trealolipídeos, produzidos por *Arthrobacter* sp. (LI *et al*., 1984); Liposan, produzido por *Candida lipolytica* (CIRIGLIANO; CARMAN, 1985); lipídeos sulfurosos, gerados por leveduras (RISTAU; WAGNER, 1993), são alguns resultados do isolamento por extração com solventes orgânicos. Os glicolipídeos produzidos por *Candida bombicola* (COOPER; PADDOCK, 1984; GÖBBERT *et al*., 1984) foram extraídos por acetato de etila gelado após adsorção por carvão. O método de precipitação por sulfato de amônio foi utilizado com sucesso no isolamento de emulsan (ROSENBERG *et al*., 1979), em biodispersan (ROSENBERG *et al*., 1988) e em um bioemulsificante produzido por uma bactéria Gram-negativa não identificada (PALEJWALA; DESAI, 1989). Um biossurfactante produzido em condições anaeróbicas por *Bacillus*

licheniformis JF-2 foi isolado pelo método de precipitação com ácido (JAVAHARI *et al.,* 1985). A remoção contínua do biossurfactante durante a fermentação foi desenvolvida por técnicas diferentes, tendo ocorrido um aumento da densidade celular no reator e a eliminação de produtos inibidores, resultando daí um acréscimo significativo no rendimento da produção (COOPER *et al.,* 1981). O método de precipitação por acetona foi empregado para isolamento de biossurfactante por ser rápido e também por permitir o monitoramento da produção máxima do biossurfactante (HEALY *et al.,* 1996). Outras técnicas de isolamento contínuas relatadas, envolvendo a adsorção em Amberlite XAD-2 seguida de purificação e liofilização, alcançaram um rendimento de 60% com 90% de pureza (REILING *et al*., 1986).

1. 4. Aplicações dos biossurfactantes

Na nova era global da industrialização, em que muitas indústrias clássicas estão sendo inovadas e redirecionadas para novas tecnologias, a biotecnologia tem um desafio que permite diversas oportunidades de pesquisa. Os rápidos desenvolvimentos na biotecnologia e o aumento da consciência ambiental entre os consumidores estão colocando os produtos biológicos na preferência do mercado (BANAT *et al*., 2000). O maior potencial de mercado para os biossurfactantes é a indústria petrolífera, tanto para a produção do petróleo quanto para a incorporação em formulações do óleo. Outras aplicações relacionadas à indústria do petróleo são a biorremediação/dispersão dos derramamentos de óleos, em terra e ao mar; e remoção/mobilização dos resíduos do óleo dos tanques de estocagem e no desenvolvimento do isolamento do petróleo (GEORGIOU *et al*., 1992; KHIRE; KHAN, 1994a, 1994b).

Recentemente, um experimento em larga escala demonstrou a eficácia, na biorremediação *in situ* no derramamento de óleo do Exxon Valdez (BRAGG *et al*., 1994). A eficiência dos

biossurfactantes na remediação de metais foi descrita por Herman *et al*. (1995), por um rhamnolipídeo com capacidade de remoção de cádmio, chumbo e zinco. A remoção de hidrocarbonetos aromáticos policíclicos foi demonstrada por Providenti *et al*. (1995) e também de outros poluentes hidrofóbicos recalcitrantes de solo contaminado por Van Dyke *et al*. (1991).

A habilidade dos biossurfactantes para emulsificar misturas de hidrocarbonetos com água foi verificada através dos trabalhos descritos por Oberbremer *et al*. (1990) e Zhang; Miller, (1995). Esta propriedade aumenta significativamente a degradação de hidrocarbonetos sendo por isso, potencialmente vantajosa para o emprego nos derramamentos de óleos (BANAT, 1995).

Na indústria de alimentos os biossurfactantes são utilizados como emulsificantes para o processamento de matérias-prima. A emulsificação é muito importante na formação da consistência e textura e também na dispersão de fases em alimentos. Um emulsificante produzido por *Candida utilis* tem sido largamente utilizados em decoração de saladas (SHEPHERD *et al*. 1995).

Os biossurfactantes encontraram uma posição adequada no mercado de cuidados pessoais devido a suas propriedades de baixa umidade e compatibilidade com a pele (BROWN, 1991).

A mistura de lipídeos sulfurosos e propileno glicol possui uma excelente compatibilidade com a pele e são produzidos comercialmente como umectantes, demonstrando a importância destes biossurfactantes para a indústria de cosméticos (DESAI; BANAT, 1997). A produção de lipídeos sulfurosos foi demonstrada por *Candida bombicola* KSM-36 com rendimento de 100-150 gl^{-1} utilizando óleo de palmeira e glicose como fontes de carbono (ITO, 1987) e por *Candida apícola*, usando glicose e óleo de girassol como substrato (STUWER, *et al.* 1987). Os lipídeos sulfurosos são comercializados por Kao Chemical Corporation, Japão, como um umectante para produtos cosméticos com marca registrada como Sofina. Esta companhia desenvolveu processos fermentativos para a produção de lipídeos sulfurosos e após um processo de esterificação em

duas etapas, o produto encontrou aplicação em batons, hidratantes para a pele e produtos para o cabelo (DESAI; BANAT, 1997).

Os biossurfactantes têm algumas aplicações terapêuticas, o efeito de inibição do crescimento do vírus da imunodeficiência humana em leucócitos, tendo sido citada na literatura (KITAMOTO *et al.,* 1993). A deficiência do surfactante pulmonar, devido a formação de um complexo de proteína-fosfolipídeo tem sido responsável pela dificuldade de respiração em crianças prematuras. O isolamento de genes para as moléculas de proteínas deste surfactante e a clonagem destes, em bactérias tem tornado possível a produção pelo processo fermentativo para sua aplicação médica (WHITE *et al.* 1985).

Os biossurfactantes são também utilizados na agricultura, como agentes ativos de superfície, necessários para a facilitar a distribuição e homogeinização dos fertilizantes, nos solos. Stanghellini e Miller (1997), avaliaram o potencial de rhamnolipídeos no controle biológico. Outras áreas de potencial aplicação são as indústrias de polpa de papel (ROSENBERG *et al*. 1989); têxtil e cerâmica (HOROWITZ; CURRIE, 1990); e ainda na imunonutrição (BENGMARK,1998).

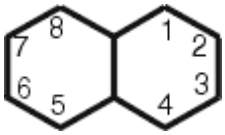
Embora as excelentes propriedades físico-químicas, como a redução da tensão superficial e atividades de emulsificação, indiquem o potencial para aplicações industriais dos biossurfactantes, os custos de produção parecem altos, cerca de 3 a 10 vezes quando comparados aos produtos sintetizados quimicamente sendo necessário maior incentivo para o estabelecimento de produtos mais econômicos.

2. Hidrocarbonetos aromáticos policíclicos (HAPs): estrutura química e propriedades

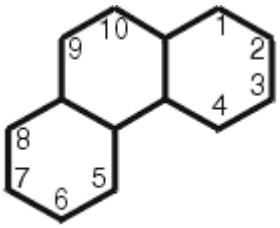
O hidrocarboneto é um dos compostos orgânicos mais simples e primitivos. É constituído somente por átomos de carbono e hidrogênio, os quais se ligam para formando estruturas lineares, cadeias ramificadas ou anéis. Os hidrocarbonetos aromáticos policíclicos constituem uma classe de compostos formados de anéis aromáticos fundidos em várias configurações estruturais demonstradas na figura 3. O grupo desses hidrocarbonetos pode ser dividido em sistemas cata-condensados e peri-condensados, dependendo da configuração dos anéis benzênicos. A estabilidade química, indicada pelo tipo de ligação entre os anéis, e a baixa solubilidade em água são as duas características principais que contribuem para a persistência dos HAPs no meio ambiente (ZANDER, 1983).

2. 1. Origem dos HAPs

Os hidrocarbonetos aromáticos policíclicos podem-se formar a partir da pirólise incompleta de materiais orgânicos encontrados em quantidades consideráveis, nos combustíveis fósseis, liberados através de processos de combustão. Os mecanismos de formação dos HAPs durante a combustão incompleta de material orgânico estão longe de um entendimento completo (LEE *et al*., 1981).



naftaleno



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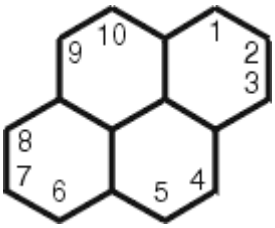
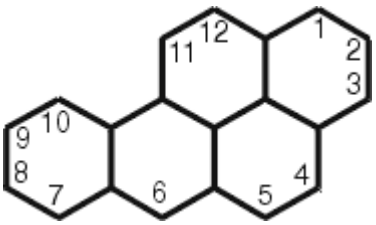


pireno



benzo[a]pireno

Figura 3. Estrutura de alguns hidrocarbonetos aromáticos policíclicos

Acredita-se que duas etapas de reações distintas estejam envolvidas no processo: a pirólise e a pirossíntese. Em altas temperaturas, os compostos orgânicos são parcialmente quebrados em moléculas menores e instáveis (pirólise), e a energia é liberada. Tais fragmentos, em sua maioria radicais, recombinam-se para produzir grandes hidrocarbonetos aromáticos relativamente estáveis (pirossíntese). No entanto, altas temperaturas e incêndios não são necessários para a aromatização da matéria orgânica. Por exemplo, os HAPs do petróleo foram formados durante milhões de anos em sedimentos que estavam sob temperaturas entre 100 e 150°C. (LEE *et al.,* 1981).

As fontes ambientais dos HAPs incluem usinas elétricas; sistemas de aquecimento doméstico à base de queima de óleo, carvão ou madeira, gasolina, máquinas a diesel, incineração de resíduos, atividades industriais diversas, fumaça de cigarro, incêndios florestais e queimadas em áreas agrícolas (FINLAYSON-PITTS; PITTS JR.,1997).

Em especial os processos de refinamento de petróleo contribuem para descargas localizadas de HAPs no ambiente, através de efluentes industriais de processamentos de liquefação e gaseificação de carvão e derramamentos acidentais de petróleo cru e refinado. Há também distribuição de HAPs a partir da atmosfera para a vegetação, o solo, a água e os sedimentos (CERNIGLIA, 1992).

Wagrowski e Hites (1997) relataram que o total de sobrecarga de HAPs nas amostras de vegetação da área rural foi em média 10 vezes menor do que nas amostras de vegetação da área urbana, confirmando que a carga de HAPs na atmosfera é maior nas regiões próximas às supostas fontes.

2. 2. Importância ambiental dos HAPs

Os hidrocarbonetos aromáticos policíclicos são compostos hidrofóbicos. Sua persistência no meio ambiente deve-se principalmente à sua baixa solubilidade em água e alta estabilidade eletroquímica. Os HAPs de alto peso molecular exibem maior persistência ambiental do que os HAPs de peso molecular mais baixo, devido ao aumento na hidrofobicidade e na estabilização da ressonância em decorrência do aumento de massa. Evidências sugerem que a hidrofobicidade, persistência ambiental e genotoxicidade dos HAPs aumentam de acordo com o tamanho da molécula do composto, ou seja, de acordo com a quantidade de anéis benzênicos fundidos (CERNIGLIA, 1992).

A relação entre o aumento do número de anéis benzeno e a persistência ambiental dos HAPs é consistente com os estudos correlacionando a taxa de biodegradação e tamanho da molécula em resultados obtidos por Bossert; Bartha (1986) e Heitkamp; Cerniglia (1987).

Da combinação das inúmeras fontes naturais e antropogênicas de hidrocarbonetos com o fenômeno do transporte global resulta a distribuição mundial desses compostos. A concentração de HAPs em uma dada área pode variar amplamente, dependendo do nível de desenvolvimento industrial e dos processos de transporte observados no local (KANALY; HARAYAMA, 2000).

Cerniglia (1992) relatou que as concentrações de HAPs no solo variam de 0,005 mg/g em áreas em desenvolvimento a 1800 mg/g nas proximidades de uma refinaria.

A combustão envolvendo compostos químicos orgânicos pode produzir misturas complexas de poluentes orgânicos semivoláteis, inclusive HAPs (WHEATLEY *et al.,* 1993; MEHARG *et al*., 1998).

O potencial de dispersão desses poluentes e a deposição de fuligem gerada por grandes queimadas podem ser consideráveis (GHONIEM *et al*., 1993; MEHARG; OSBORNE, 1995).

O monitoramento atmosférico do meio ambiente para esses compostos orgânicos semivoláteis revela a dependência das propriedades físico-químicas de tais compostos com respeito a suas afinidades para fases gasosas e sólidas (HARRISON; JONES, 1995; TREMOLADA *et al.,* 1996).

A contaminação do solo, das colheitas e mudas, a partir da decomposição atmosférica, é a principal rota de entrada dos compostos semivoláteis liberados por acidentes na cadeia agrícola e de alimentos (LIJINSKI, 1991; MEHARG *et al*., 1998).

Portanto, a distribuição global dos HAPs depende de suas origens. No Brasil, estudos realizados nas proximidades de Manaus (AM) detectaram HAPs em altos níveis, originados de processos de combustão. A exploração econômica da floresta amazônica estimula a queimada de grandes áreas florestais para a fixação de culturas agrícolas, alterando a ecologia regional. A fumaça resultante dessas queimadas pode-se propagar por grandes extensões, espalhando compostos tóxicos como os HAPs (ABAS *et al*., 1995).

Wilcke *et al*. (2003) encontraram, no solo de diferentes regiões ecológicas brasileiras, como o cerrado, a Mata Atlântica e a caatinga, uma mistura de 20 diferentes HAPs, devido principalmente, à queima de matas. Os hidrocarbonetos naftaleno e perileno foram produzidos ou acumulados, respectivamente, em ninhos de cupins na região amazônica e na maioria dos ninhos em todo o Brasil.

2. 2. 1. Toxicidade

Diversos hidrocarbonetos aromáticos policíclicos apresentam propriedades tóxicas, mutagênicas e/ou carcinogênicas (MASTRANGELO *et al*., 1996; GOLDMAN *et al*., 2001).

O contaminação por HAPs através da inalação, ingestão ou contato com a pele de fuligem, gases, alcatrão, resíduos de petróleo, ar ou alimentos contaminados é especialmente preocupante devido à toxidade desses compostos. Vários estudos a respeito do potencial carcinogênico dos HAPs mostram que essas substâncias adquirem tal característica após a ativação do aceptor celular, mecanismo que pode ser observado no benzo[a]pireno: inicialmente, um epóxido areno é formado por uma mono-oxigenase do citocromo tipo P450; o epóxido é então hidrolisado por um epóxido hidrolase a um trans-diol; em seguida, outro epóxido é formado e catalisado novamente por uma enzima P450. Esse diol-epóxido é altamente reativo para substâncias nucleofilicas e tem habilidade para reagir com o DNA (MOORTHY *et al.,* 1994; STAGEMAN *et al*., 2001).

Além disso, muitos hidrocarbonetos aromáticos policíclicos são considerados poluentes ambientais, capazes de causar efeitos prejudiciais sobre a flora e fauna, ou mesmo afetar os *habitats*. O dano ambiental causado pelos HAPs resulta da absorção e acumulação de produtos químicos tóxicos pela cadeia alimentar, o que gera problemas sérios de saúde e/ou defeitos genéticos no homem. Conseqüentemente, a Agência Americana de Proteção Ambiental (EPA) listou dezesseis HAPs como poluentes prioritários para remediação (LIU *et al*., 2001; DONKIN *et al*., 2003).

O naftaleno, com seus dois anéis de benzeno, é o composto mais simples do grupo dos HAPs. É considerado um micropoluente comum na água potável. Sua toxicidade e atividade teratogênica foram relatadas em animais de laboratório. Alterações dermatológicas e

oftalmológicas foram observadas em trabalhadores ocupacionalmente expostos ao naftaleno (MASTRANGELO *et al*., 1996; GOLDMAN *et al*., 2001).

O fenantreno é um HAP constituído por três anéis aromáticos fundidos. Esse HAP é tido como fotossensibilizante para a pele humana. Sob condições específicas, pode ser mutagênico para bactérias (MASTRANGELO *et al*., 1996).

O pireno, com quatro anéis aromáticos é o HAP mais abundante no meio ambiente. Embora não seja genotóxico, é utilizado como indicador no monitoramento de sítios contaminados, visto que sua estrutura molecular é encontrada em HAPs potencialmente carcinogênicos (SACK *et al*., 1997).

O benzo[a]pireno é um HAP que tem sido estudado extensivamente por muitos anos, devido à sua potente genotoxidade (SUTHERLAND *et al.,* 1995) e propriedades imunotóxicas (DAVILA, 1999).

Por tudo isso, existe um grande interesse em se determinar o destino dos HAPs no meio ambiente. Os objetivos das pesquisas científicas nesse sentido tem sido avaliar o risco da exposição do homem a esses compostos (ANGERER *et al*., 1997) e reduzir eficientemente os elevados níveis de HAPs em sítios contaminados.

2. 3. Degradação microbiológica

Microrganismos são utilizados para degradar compostos perigosos em substâncias inofensivas, tais como dióxido de carbono e água. As reações de transformação microbiológica são mediadas por fungos e bactérias, responsáveis pela grande maioria dos processos de transformação biológica. Esses microrganismos podem ser responsáveis pelo desaparecimento de contaminantes orgânicos do meio ambiente. A primeira etapa na degradação microbiológica dos

HAPs envolve a ação de enzimas chamadas oxigenases, produzidas pelos microrganismos que catalisam as reações de fixação do oxigênio. Há dois grupos de oxigenases: as mono-oxigenases e as di-oxigenases. Os fungos e os mamíferos geralmente produzem mono-oxigenases, as quais incorporam um átomo de oxigênio a cada dois átomos de carbono, resultando daí a formação de intermediários (WILSON; JONES, 1993).

No caso das bactérias, a etapa inicial ocorre via oxidação do hidrocarboneto para um di-idrodiol, através de um sistema de componentes multienzimáticos. Esses intermediários di-idroxilato podem então ser processados por uma ou outra via de clivagem (orto ou meta-clivagem), produzindo intermediários principais, como o protocatecuato e catecol, os quais se convertem em intermediários do ciclo do ácido tricarboxílico (KANALY; HARAYAMA, 2000). A habilidade dos microrganismos (bactérias, fungos) em utilizar hidrocarbonetos como única fonte de energia em seu metabolismo foi relatada por Zobell (1946) e Bushnell e Haas (1940). Na última década, as pesquisas sobre a biodegradação da HAPs constituídos por mais de três anéis têm avançado significativamente.

Vinte e um anos após a revisão clássica realizada por Zobell, um acidente com navio petroleiro provocou derramamento de óleo e atraiu a atenção da comunidade científica para os problemas da poluição ambiental (ATLAS, 1981).

- Microrganismos que degradam hidrocarbonetos aromáticos policíclicos com dois anéis aromáticos por exemplo, o naftaleno:

Bactérias

Existe um grande número de bactérias que degradam o naftaleno e que já foram estudados. Duas amostras de bactérias com habilidade para utilizar naftaleno como única fonte de

carbono e energia foram isoladas de sedimento contaminado com creosoto. Essas amostras foram incubadas com HAP em meio de cultura com água do mar artificial. As bactérias foram identificadas como pertencentes ao gênero *Pseudomonas* e *Burkholderia* (HEDLUND *et al.,* 1999).

Mais de cem amostras de bactérias com capacidade para utilizar naftaleno como fonte de carbono foram isoladas de sedimentos marinhos de uma área poluída. A maioria dessas amostras pertence ao gênero *Pseudomonas*. Sete isolados apresentaram novos biovares pertencentes às espécies *Pseudomonas testoteroni* e *P. stutzeri*, que degradam hidrocarbonetos aromáticos (GARCIA-VALDÉS *et al*., 1988).

Fungos

Cunninghamella elegans foi cultivada em caldo Sabouraud dextrose na presença de naftaleno, produzindo seis metabólitos os quais foram isolados e identificados por técnicas químicas convencionais. Os resultados sugerem que esse fungo oxida naftaleno através de uma seqüência de reações similares às que ocorrem no metabolismo desse hidrocarboneto pelos mamíferos (CERNIGLIA; GIBSON, 1977).

Seis espécies de leveduras foram examinadas em sua habilidade para metabolizar naftaleno, bifenil e benzo[a]pireno. Todos os organismos testados oxidaram esses compostos aromáticos. As culturas testadas foram das leveduras *Debaryomyces hansenii*, *Candida tropicalis*, *C. maltosa*, *C. guilliermondii*, além de duas amostras de *C. lipolytica*, 37-1 e 78-003. *C. lipolytica* oxidou os HAPs em grande extensão, por isso foi o organismo escolhido para estudos posteriores (CERNIGLIA; CROW, 1981).

- Microrganismos que degradam hidrocarbonetos aromáticos policíclicos com três anéis aromáticos por exemplo, o fenantreno:

Bactérias

O isolamento de bactérias degradadoras de fenantreno de sedimentos de zona portuária em Boston resultou num total de 423 isolados em um estudo de mais de dois anos. Essas bactérias foram caracterizadas, fenotipicamente, nos gêneros *Pseudomonas*, *Burkolderia*, *Sphingomonas*, *Flavobacter*, *Vibrio* e *Mycobacterium*. Técnicas moleculares revelaram que a comunidade dessas bactérias degradadoras de fenantreno nos sedimentos é bastante diversificada, e que a sua estrutura sofreu modificações significativas no decorrer do tempo. Além disso, os genes predominantes que regulavam a degradação do fenantreno na comunidade não estavam bem caracterizados (BERARDESCO *et al.,* 1998).

Boldrin et al. (1993) isolaram uma amostra de *Mycobacterium* sp. amostra BBI de um antigo local de gaseificação de carvão. Essa amostra mostrou habilidade para utilizar fenantreno, pireno ou fluoranteno como única fonte de carbono e energia. As taxas de crescimento foram de 0,069, 0,056 e 0,040 h^{-1}, respectivamente.

Bactérias com capacidade para degradar fenantreno foram isoladas em amostras de água e solo contaminadas com creosoto e óleo diesel. Nesse estudo, modificou-se a técnica de isolamento em placas com agarose resfriado: adicionaram-se bactérias e partículas finas de fenantreno, que foram distribuídas sobre a superfície do ágar anteriormente solidificado. As bactérias degradadoras mostraram halos ao redor das colônias, demonstrando o desaparecimento do fenantreno (BOGART; HEMMINGSEN, 1992).

Fungos

O potencial dos fungos na oxidação dos HAPs já é bem conhecido. *Cunninghamella elegans*, estudada em trabalhos realizados por Cerniglia *et al.* (1985), e o fungo da podridão branca *Pleorotus ostreatus*, que metabolizou 94% do fenantreno adicionado ao meio rico para basidiomycetos, após 11 dias de crescimento (BEZALEL *et al.*, 1996).

Sack *et al*. (1997) realizaram um estudo com cinco diferentes fungos da podridão da madeira na degradação do fenantreno e do pireno, tendo sido os fungos cultivados em um substrato natural. Os HAPs foram adsorvidos em palha de trigo. *Trametes versicolor* e *Kuehneromyces mutabilis* mineralizaram respectivamente o fenantreno 15,5 e 5,0% em 63 dias. *Laetiporus sulphureus* e *Agrocybe aegerita* mineralizaram 10,7 e 3,7% do fenantreno, respectivamente. Com o fungo *Flammulina velutipes* não foi detectada mineralização.

Amostra de *Aspergillus niger* isolado de solo contaminando com HAPs foi examinada em seu potencial para degradar fenantreno e pireno. Essa amostra metabolizou ambos os compostos, produzindo metabólicos menores: 1-fenantrol, 2-fenantrol e 1-pirenol (SACK *et al*., 1997).

- Microrganismos que degradam hidrocarbonetos aromáticos policíclicos com quatro anéis aromáticos por exemplo, o pireno:

Bactérias

A degradação de três HAPs – pireno, benzo[a]antraceno e benzo[a]pireno – foi relatada por Schneider *et al*., em 1996. Utilizou-se no estudo a bactéria *Mycobacterium* sp. amostra RJGII-135, isolada do solo de um sítio abandonado contaminado por carvão, através de técnicas

de enriquecimento. As vias de degradação destes três compostos pela bactéria, confirmam os processos enzimáticos de di-oxigenases descritos previamente, para outra bactérias.

A investigação da degradação e mineralização de HAPs de alto peso molecular, tais como pireno, criseno, benzo[a]pireno e benzo[a]antraceno e dibenzo[a, h] antraceno, foi realizada por Boonchan *et al.* (2000), através da utilização de um consórcio da bactéria *Stenotrophomonas maltophilia* e do fungo *Penicillium janthinellum*.

Fungos

Estudo realizado para isolamento de fungos com habilidade para degradar pireno foi realizado em sedimentos contaminados com HAPs. Foram isolados dez fungos degradadores do pireno, nove deles ainda não relatados pela literatura: Zygomycetes – *Mucor racemosus*, *M. racemosus var. sphaerosporus*; Deuteromycetes – *Gliocladium virens*, *Penicillium simplicissimum*, *P. janthinellum*, *Phialophora alba*, *P. hoffmannii*, *Trichoderma harzianum;* Dematiaceae – *Scopulariopsis brumptii*, e o Sphaeropsidale *Coniothyrium fuckelii* (RAVELET *et al*., 2000). O fungo da podridão branca *Cariolopsis gallica* (UAMH 8260) produziu a enzima lacase, que metabolizou o pireno (PICKARD *et al*, 1999).

A habilidade de diferentes espécies e amostras do gênero *Crinipellis*, *Marasmius* e *Marasmiellus* para metabolizar o pireno foi investigada. Foram detectados, nesse trabalho, vários metabólicos dependentes do meio de cultura utilizado e de cada amostra específica de diferentes espécies de Basidiomycetes (LANGE *et al*., 1996).

- Microrganismos que degradam hidrocarbonetos aromáticos policíclicos com cinco anéis aromáticos por exemplo, o benzo[a]pireno:

Bactérias

Na literatura, poucos são os relatos que descrevem a degradação de benzo[a]pireno por cultura de bactérias puras ou mistas (TRZESICKA-MLYNARZ; WARD, 1995; SCHNEIDER *et al.*, 1996; JUHASZ *et al*., 1997). Além disso, existem muito poucos trabalhos a respeito da extensiva mineralização do benzo[a]pireno por bactérias. Ye *et al.* (1996) demonstraram que uma suspensão por células de *Sphingomonas paucimobilis* (EPA 505) converteu co-metabolicamente 10.mg l^{-1} de [7-^{14}C] benzo[a]pireno a 28% $^{14}CO_2$ em 48 horas. Recentemente, Boonchan *et al.* (2000) descreveram uma cultura mista de bactéria-fungo que mineralizou co-metabolicamente 50.mg l^{-1} de [7-^{14}C] benzo[a]pireno a 58,1% de CO_2 em 56 dias, na presença de 250 mg.l^{-1} de pireno. Ainda mais notável foi a mineralização desse composto em 25,5% pelo consórcio de fungo-bactéria, como única fonte de carbono e energia sob as mesmas condições.

Fungos

O metabolismo de benzo[a]pireno por culturas de fungos da podridão branca já foi demonstrado previamente (HAEMMERLI *et al*., 1986). O benzo[a]pireno foi rapidamente oxidado em compostos polares solúveis em água pelo fungo da podridão branca *Bjerkandera* sp. (SBOS55). A maioria desses metabólicos foi mineralizada pela comunidade microbiana nativa não adaptada a HAPs, sob condições aeróbicas (KOTERMANN *et al*., 1998).

O benzo[a]pireno pode ser removido do meio ambiente através da ação de fungos. Vários estudos realizados nos últimos cinco anos descreveram a biodegradação desse composto por fungos da podridão branca, através do sistema enzimático ligninolítico extracelular (lignina peroxidase e manganês peroxidase). *Trametes versicolor* oxidou *in vitro*, por meio da isoenzima

lacase, os HAPs benzo[a]pireno e antraceno (COLLINS *et al*., 1996). *Stropharia coronilla*, um Basidiomycete que decompõe matéria orgânica, mostrou capacidade para metabolizar e mineralizar benzo[a]pireno em cultura líquida, suplementada com íons manganês (Mn^{2+}), o que estimulou consideravelmente o efeito da enzima ligninolítica manganês peroxidase (STEFFEN *et al*., 2003).

2. 3. 1. Biorremediação

Durante a última década, muitos microrganismos foram isolados e caracterizados conforme a sua habilidade para degradar diferentes HAPs. Assim, novas vias de degradação foram elucidadas. Para melhorar a biodegradação de compostos tóxicos no meio ambiente, diferentes estratégias podem ser consideradas, como, por exemplo, a quimiotaxia (GRIM; HARWOOD, 1999). Outra estratégia seria a utilização de microrganismos geneticamente modificados embora os relatos sobre esse assunto ainda sejam escassos, estudos têm sido realizados, visando o melhoramento nas taxas de mineralização. A caracterização genética dos genes *phn* da amostra *Burkholderia* sp. RP 007 levou ao entendimento do processo da degradação de HAPs por bactérias (TAKIZAWA *et al*., 1999).

Recentemente, os genes da dioxigenase de *Mycobacterium* sp. amostra PYR-1 foram clonados, subclonados e superexpressados em *Escherichia coli*, através do sistema pBAD/ThioFusion. A funcionalidade dos genes para degradação do hidrocarboneto foi confirmada em um clone do fago contendo os dois genes que codificam as subunidades di-oxigenases (KHAN *et al*., 2001).

As pesquisas sobre biorremediação de HAPs e outros compostos similares no meio ambiente têm sido discutidas. Os HAPs podem ser degradados através de uma grande

diversidade de bactérias que existem naturalmente no solo, e que provaram ser eficientes no campo (WILSON; JONES, 1993). A biorremediação por intermédio de microrganismos naturais foi o principal mecanismo de remoção do óleo derramado pelo Exxon Valdez no Alasca (SUGAI *et al*., 1997).

Um consórcio de cinco amostras selvagens de bactérias foi selecionado para ser aplicado em sítios contaminados por HAPs em uma refinaria na Índia. Nesse estudo, foram avaliados os níveis de inóculo e as taxas de sobrevivência dos microrganismos introduzidos com e sem a suplementação com nutrientes. A aplicação do consórcio de bactérias e nutrientes, resultaram em máxima biodegradação de hidrocarbonetos derivados do petróleo (MISHRA *et al*., 2001).

A remediação microbiana de HAPs em solos contaminados por petróleo é uma tecnologia emergente, envolvendo a aplicação de biossurfactantes. A adição do biossurfactante estimula a população de microrganismos selvagens a degradarem os hidrocarbonetos em altas taxas, maiores do que as alcançadas através da adição somente de nutrientes (BANAT, 1993, 1995).

Vários trabalhos têm demonstrado os efeitos benéficos dos biossurfactantes sobre a taxa de oxidação dos hidrocarbonetos, devido ao aumento da bio-acessibilidade destes aos microrganismos (NOORDMAN *et al*., 1998; DOONG, LEI, 2003). Pesquisas adicionais ainda serão necessárias para explorar as interações microbiológicas dentro dos consórcios que degradam os HAPs, os mecanismos regulatórios da degradação dos HAPs com vários anéis em sua estrutura, bem como a biodegradação co-metabólica dos HAPs. Novas abordagens e recentes avanços na biologia molecular, poderão auxiliar na detecção de microrganismos degradadores de amostras ambientais (TIMMIS; PIEPER, 1999).

A biorremediação é considerada ainda uma tecnologia em desenvolvimento (WATANABE, 2001). Por conseguinte, são essenciais o levantamento de informações sobre as populações microbianas degradadoras, o auxílio de propostas moleculares ecológicas e o

conhecimento da fisiologia e genética desses microorganismos, a fim de que se estabeleçam projetos de biorremediação ambientalmente seguros.

3. Biossorção

O processo de sorção consiste no acúmulo de substâncias químicas na superfície (adsorção) ou no interior (absorção) da célula microbiana (ALEXANDER, 1994). A biossorção é a absorção ou acumulação de produtos químicos pela massa microbiana. A biossorção envolve a combinação de mecanismos de transporte ativo e passivo, iniciando-se com a difusão do componente adsorvido pela superfície da célula. Quando o componente se difunde na superfície celular, ele se liga a sítios que exibem algumas afinidades químicas para o componente. Geralmente, tal adsorção é rápida, reversível, não constituindo fator limitante na cinética da biorremoção quando processada com células dispersas. A biossorção é freqüentemente seguida de um processo de ligação lento, no qual componentes adicionais são ligados, em regra irreversivelmente (AKSU; TEZER, 2000).

A sorção é considerada um dos processos mais significativos para remoção dos contaminantes orgânicos em tratamentos de sistemas de resíduos, juntamente com a biodegradação e a destruição (JACOBSON *et al*., 1993). Existem alguns estudos de sorção de poluentes orgânicos em solos ou sedimentos, mas pouquíssimos experimentos foram realizados com biomassa microbiana (BELL; TSEZOS, 1988). Muitos poluentes orgânicos perigosos têm sido identificados nos efluentes e lodos dos serviços de tratamento de resíduos industriais. Esses poluentes são, na maioria das vezes, recalcitrantes e provenientes de instalações industriais. A biodegradação poderia ser um processo viável para remover alguns desses compostos, entretanto os produtos derivados da degradação podem ser também perigosos. Outro mecanismo de

remoção seria a acumulação das substâncias químicas através de biomassa microbiana (BELL; TSEZOS, 1988).

Existem vários mecanismos distintos que podem promover a acumulação de hidrocarbonetos aromáticos policíclicos em resíduos biológicos. Dentre esses mecanismos, incluem-se a absorção dentro das estruturas lipídicas de bactérias, a sorção sobre as estruturas polissacarídicas localizadas no exterior da célula e a ligação química com as proteínas e ácidos nucléicos da bactéria (MORETTI; NEUFELD, 1989).

O processo de biossorção está sendo bastante desenvolvido para várias aplicações nas diversas áreas de biorremediação, a fim de que se identifiquem adsorventes biológicos eficientes e de baixo custo. Os adsorventes biológicos oferecem viabilidade técnica e são economicamente atrativos (AKSU; DÖNMEZ, 2003). A capacidade de adsorção de diversos microrganismos como algas, bactérias e fungos está bem relatada na literatura, o que pode ser potencialmente explorado na biotecnologia ambiental (WHITE; GADD, 2000). Fungos são microrganismos que crescem com facilidade, produzem alto rendimento de biomassa e ao mesmo tempo podem ser manipulados genética e morfologicamente. Esses organismos são largamente utilizados em uma grande variedade de processos industriais de grande escala. Sua biomassa poderia ser usada como subproduto de baixo custo em quantidades substanciais (KAPOOR; VIRARAGHAVAN, 1995). Alternativamente, poderia também ser cultivada utilizando-se processos fermentativos simples e meios de cultura de baixo custo (KUYUCAK, 1990).

A levedura Ascomycetes *Candida lipolytica* é um dos organismos mais extensivamente estudados, sendo considerada uma levedura "não convencional" (BARTH; GAILLARDIN, 1997). Tem demonstrado alta capacidade de acumulação e adsorção de compostos orgânicos tóxicos, como metais pesados e corantes (DÖNMEZ; AKSU, 2001; AKSU; DÖNMEZ, 2003).

A biossorção de 1, 2, 3, 4-tetraclorodibenzo-p-dioxina, dibenzofurano e policlorados pela bactéria *Bacillus pumilus* foi investigada em trabalho que visava determinar a importância do microrganismo como potencial agente na transferência de moléculas no ambiente. Os resultados do estudo indicaram que a biomassa morta dessa bactéria removeu moléculas do meio ambiente mais efetivamente que as células vivas (HONG *et al.,* 2000). Dando prosseguimento a essa análise, os pesquisadores focalizaram a sorção de poluentes aromáticos halogenados por compostos biológicos liberados por *Bacillus pumilus*. Quando essa bactéria é exposta a alta temperatura, libera uma proteína e carboidratos, exclusivamente. Observou-se que os poluentes foram adsorvidos consideravelmente não somente pelas células, mas também pela proteína liberada. Os resultados obtidos nesse estudo são consistentes com adsorção via mecanismo físico-químico passivo (CHOI *et al*., 2003).

A eficiência de sete espécies de microalgas na remoção do pireno foi relatada por Lei *et al.* (2002). Para todas as espécies estudadas, a remoção foi muito rápida, ocorrendo no intervalo entre as 3 e 6 horas iniciais do tratamento. Não foi observada diferença significativa entre células vivas e mortas de três espécies, o que indica que a remoção inicial deveu-se a biossorção físico-química passiva. Demonstrou-se ainda que mais de 65% do pireno adsorvido estavam ligados a materiais da parede celular de *Selenastrum capricornutum*, o que sugere que o principal sítio de ligação localizou-se na parede celular. A remoção do pireno foi também dependente da concentração da biomassa da alga utilizada. Por outro lado, a biossorção do pireno ocorreu ainda por acumulação e transformação dentro das células vivas. Após sete dias de cultivo de *S. capricornutum*, o pireno não foi mais detectado no meio de cultura e nem nas células da alga, o que sugere que o composto deve ter sido completamente transformado por essa espécie.

A levedura *Trichosporon cutaneum* (R57) mostrou habilidade para crescer e utilizar alguns compostos tóxicos, como fenol, acetofenona, acetona, α-metilestireno, ácido benzóico,

dimetilcarbinol, metanol e isopropilbenzeno. Estudo foi realizado com uma solução-modelo, que incluía todos os contaminantes mencionados acima, além de células em suspensão e imobilizadas. Os resultados do estudo – comparados com curvas de crescimento em dois tipos de resíduos de uma estação de purificação com lodo ativado e águas tratadas de uma refinaria de petróleo (incluindo resíduos de uma indústria de produção de fenol) – revelaram que *Trichosporon cutaneum* (R57) pode utilizar uma larga escala de compostos tóxicos simultaneamente. Por sua vez, as células imobilizadas mostraram uma alta habilidade em degradar, se comparadas às células em suspensão (GODJEVARGOVA *et al*., 2003).

A biomassa de *Aspergillus niger* foi estudada para uso na remoção de fenol em solução aquosa. Cinco tipos de biomassa não viável pré-tratada foram utilizados como biossorventes. A dessorção do fenol em água destilada deionizada atingiu um percentual de 5%, sugerindo uma forte biossorção pela biomassa. Os resultados deixaram claro que 66% do fenol foram removidos, através do funcionamento da coluna de polisulfona em uma concentração inicial de 1000 $\mu g\ l^{-1}$ do fenol (RAO; VIRARAGHAVAN, 2002).

A degradação microbiana de fenol (300–1300mg/l^{-1}), p-nitrofenol (50–300 mg/l^{-1}) e fenantreno (50–300 mg/l^{-1}) adsorvidos em carvão ativado foi estudada. A combinação de sorção física e degradação biológica no carbono ativado foi comparada com o desempenho da degradação de uma suspensão mista de bactérias isoladas de solo contaminado com resíduos de pesticidas. Os perfis de degradação quase iguais foram obtidos nas culturas com carvão ativado para os três compostos citados. Contudo, nas concentrações mais altas, a degradação foi inibida ou cessou completamente, embora culturas de bactérias mistas especializadas, adaptadas a altas concentrações de compostos tóxicos, tenham sido usadas em cada caso. Os autores desse estudo focalizam a aplicabilidade do carbono ativado em processos contínuos com bactérias especializadas, para tratamento de produtos químicos tóxicos (ABU-SALAH *et al*., 1996).

O objetivo do trabalho realizado por Stringfellow e Alvarez-Cohen (1999) foi investigar a sorção de HAP por biomassa bacteriana e examinar a relação entre biossorção e biodegradação de HAP. Nesse estudo, o fenantreno foi utilizado como modelo de HAP para a biossorção, enquanto o pireno e o fluorantreno foram empregados como compostos modelares nos estudos de biodegradação. Observou-se que a biossorção do fenantreno variou de acordo com o gênero e a espécie da bactéria. As capacidades de sorção medidas eram reproduzíveis e aparentemente representaram a sorção superficial, fundamentada na evidente competição entre naftaleno e fenantreno por sítios de sorção. Os resultados desse estudo sugerem que, embora a biossorção possa diminuir a taxa de biodegradação de HAP em curto prazo, ela pode também resultar na remoção de HAP dos resíduos e na retenção do composto no sistema de tratamento, onde finalmente ele será biodegradado.

Nos últimos anos, numerosos estudos focalizaram, sobretudo, a remoção de metais e diversos tipos de corantes por meio de biomassa fúngica, viva ou morta, com ou sem pré-tratamento químico ou físico. Tais estudos geraram um aumento de interesse no potencial da biomassa de fungos como biossorventes. Em um estudo comparativo utilizando nove espécies de leveduras, *Candida lipolytica* promoveu a taxa mais alta na remoção de corantes tóxicos (AKSU; DÖNMEZ, 2003). No entanto, ainda são poucas as publicações a respeito da interação dessa biomassa com tóxicos orgânicos. Neste trabalho, a utilização de *Candida lipolytica* se justifica pela sua comprovada habilidade de biossorção.

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CAPÍTULO I

New Bioemulsifiers Produced by *Candida lipolytica* using D-Glucose and Babassu Oil as Carbon Sources

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NEW BIOEMULSIFIERS PRODUCED BY *CANDIDA LIPOLYTICA* USING D-GLUCOSE AND BABASSU OIL AS CARBON SOURCES

Mabel H. Vance-Harrop[1,2]; Norma B. de Gusmão[2,3]; Galba Maria de Campos-Takaki[2,4,5]*

[1]Laboratório de Apoio Animal, Ministério da Agricultura Pecuária e do Abastecimento; [2]Doutorado em Biologia de Fungos, Universidade Federal de Pernambuco; [3]Departamento de Antibióticos, Universidade Federal de Pernambuco; [4]Departamento de Química, [5]Núcleo de Pesquisas em Ciências Ambientais, Universidade Católica de Pernambuco, Recife, PE, Brasil

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ABSTRACT

Candida lipolytica IA 1055 produced extracellular biosurfactants with emulsification activity by fermentation using babassu oil and D-glucose as carbon sources. Natural seawater diluted at 50% supplemented with urea, ammonium sulfate, and phosphate was used as economic basal medium. The best results were achieved with the **YSW-B_2** medium, which contained urea, ammonium sulfate, and babassu oil and with **YSW-B_3** medium, which contained urea, ammonium sulfate, phosphate, and babassu oil, kept under fed batch fermentation for 60 hours with 5% of babassu oil. For the two media, the maximum specific growth rates were 0.02 h^{-1} and 0.04 h^{-1}; the generation times were 34.6 h^{-1} and 17.3 h^{-1}, and the emulsification activities were 0.666 and 0.158 units, respectively. The molecules of these new bioemulsifiers were contituted of carbohydrates, proteins and lipids.

Keywords: Bioemulsifier, *Candida lipolytica*, seawater, babassu oil

Biosurfactants are amphiphilic molecules consisting of a hydrophilic and a hydrophobic domains. These compounds are capable of reducing surface and interfacial tension between liquids, solids and gases, allowing them to mix or disperse readily as emulsions in water or other liquids. Microbial compounds that are capable to exhibit particularly high surface activity and emulsifying activity are classified as biosurfactants (1). A number of microorganisms are excellent sources of potentially useful amphipatic biopolymers, for instance *Acinetobacter radioresistens* (5); *Candida bombicola* (9,13), and *Yarrowia lipolytica* (14).

Bioemulsifiers are biodegradable and can be produced from renewable and cheap subtracts. Native vegetable oils have been used as carbon source and seawater as media component for production of biosurfactants by *Candida lipolytica*. This paper reports the results attained on bioemulsifier production through the use of economic subtracts in seawater media supplemented with babassu oil, and D-glucose as control.

Candida lipolytica IA 1055 was obtained from the culture collection of the Departamento of Antibióticos of Universidade Federal de Pernambuco. Stock cultures of the organism were maintained at 4ºC on Yeast Mold Agar slants containing yeast extract (0.3%); malt extract (0.3%); D-glucose (1.0%); tryptone (0.5%); and agar (1.5%).

Carbon sources utilized throughout the study were 5% babassu oil and 1% D-glucose, added to a basal medium called Yeast Salt Water (YSW). Growth media were prepared as follows: **YSW-G** – ammonium sulfate 0.1gl^{-1}, D-glucose 1gl^{-1}; **YSW-G_1** – ammonium sulfate 0.1gl^{-1}, urea 0.1gl^{-1}, D-glucose 1gl^{-1}; **YSW-B_2** –urea 0.25gl^{-1}; acid phosphate of potassium 1.36gl^{-1}, babassu oil 5 ml^{-1}; and **YSW-B_3** – ammonium sulfate 0.1gl^{-1}, urea 0.25gl^{-1}, and acid phosphate of potassium 1.36gl^{-1}, and babassu oil 5 ml^{-1}.

The media were diluted 1:1 (v/v) in distilled water and natural seawater (NaCl final concentration 13%); the pH was set to 5.3±0.2, followed by sterilization at 121ºC for 15 minutes. The seawater used as basal medium was collected in Boa Viagem

* Corresponding author. Mailing address: Departamento de Química, Núcleo de Pesquisas em Ciências Ambientais, Universidade Católica de Pernambuco. Rua Nunes Machado, 42, Boa-Vista. 500050-590, Recife, PE, Brasil. E-mail: takaki@unicap.br

beach, and the salinity was determined by the Mohr-Knudsen method as described in Strickland and Parsons (11).

Cells grown in Yeast Mold Broth were used as inoculum (1%, v/v). The fermentation process was carried out in 1000ml-Erlenmeyer flasks, containing 300ml of medium. The flasks were incubated at 28ºC on an orbital shaker at 150 rpm for 240 hours. After 60 hours of fermentation, 5% of babassu oil was added to the medium.

Aliquots of 7 ml of the culture were collected every 24 hours for pH measurement using a potentiometer and for counts of viable cells, determined by spreading 0.1ml of the appropriate dilution onto Yeast Mold Agar. Colony counts were performed after incubation at 28ºC for 48 hours.

The specific growth rate was measured according to Pirt (8).

In order to determine the emulsification activity, aliquots of 2 ml of the cultures submitted to filtration through a 0.22 µm Millipore membrane filter were mixed with 2ml of 0.1M sodium acetate buffer (pH 3.0) and 1ml of n-hexadecane in a screw-capped tube. The mixture was then homogeneized in a vortex for 2 minutes at 25ºC. After 10 minutes, the absorbance was measured at 540nm in a spectrophotometer. The blank consisted in 2ml of sterile YSW medium. One unit of emulsification activity was defined as the amount of emulsifier that resulted in an absorbance of 1.0 at 540nm (2).

For partial purification of the bioemulsifier, yeasts cells were removed from bioemulsifier-containing media, by filtration through a Whatman n° 1 paper and then through a 0.22 µm Millipore membrane filter.

The cell-free filtrate was dialyzed against deionized water, (five changes) and then concentrated by lyophilization. The concentrate was treated twice with chloroform-methanol (2:1, v/v) at 25ºC. The solvents were removed by evaporation. The white precipitate formed in the aqueous phase was collected in Whatman no. 42 filter paper and dried. The dry material was redissolved in ultra pure water for analytical determinations.

The bioemulsifiers produced by *C. lipolytica* were submitted to determination of the total amount of carbohydrates, proteins and lipids, using colorimetric methods (LABTEST Diagnostic®-Brazil).

After 41 hours, the cell concentration in YSW-G medium was $1.0x10^6$ CFU/ml. After 50 hours, a diauxic behavior was observed. The number of cells after 89 hours decreased to $3.2x10^2$ CFU/ml and the specific growth rate was $\mu_{max}=0.04h^{-1}$. During fermentation course, the pH dropped from 4.8 after 17 hours to 2.7 at the end of the growth, suggesting that fermentation is controlled by pH (Fig. A). The cell concentration in YSW-G_1 medium, which corresponds to YSW-G medium plus 1% urea, after 23 hours of cultivation was $1.1x10^6$ CFU/ml. The peak was obtained after 168 hours of fermentation, with $6.0x10^6$ CFU/ml. There was a growth decrease after 192 hours. The kinetics parameters were a maximum specific growth rate of $\mu_{max}=0.05h^{-1}$ and generation time of 13.8 hours. After 23 hours of fermentation the pH was 8.04, and 7.04 in the end of the process, and the emulsification activities were 0.030, and 1.717 units, respectively (Fig. 1B).

The growth decrease of *C. lipolytica* was probably due to the addition of urea, which increased the pH of the medium. Cooper and Paddock (3) observed that *Torulopsis bombicola* growth decreased when urea was used instead of yeast extract in media with glucose and vegetable oils. Other microbial emulsifiers (sophorose lipids) have been synthesized by degradation of insoluble substrates such as vegetable oils or soluble substrates, like carbohydrates, as carbon sources, utilizing inexpensive media (6,12,13).

In this study, the YSW-G medium was the most effective for growth of *Candida lipolytica*. This result with the medium YSW supplemented with glucose without urea is corroborated by Cooper and Paddock (3,10).

After 48 hours of cultivation, $3.1x10^2$ viable cells were observed in YSW-B_2 medium. The maximum number of cells was attained after 144 hours, with $1.8x10^7$ CFU/ml. The pH was 7.5 in the first stage (48 h) of the fermentation, and decreased to 5.6 in the end. The maximum specific growth rate was $\mu_{max}=0.02h^{-1}$, the generation time was 34.6 h^{-1}, and the emulsification activity was 0.666 units (Fig. 1C).

After 64 hours of fermentation *C. lipolytica* cultivated in the medium YSW-B_3 showed $2.8x10^5$ CFU/ml. The maximum number of viable cells occurred after 168 of cultivation. At the beginning of the process (24 h), the pH was 6.4, decreasing to 5.9 after 168 hours. A maximum specific growth rate of $\mu_{max}=0.04h^{-1}$, generation time of 17.3 h^{-1}, and 0.158 units of emulsification activity were observed (Fig. 1D).

Johnson (4) reported that the maximum emulsification activity in *Rhodotorula glutinis* was achieved in the presence of KNO_3, followed by ammonium salt and urea. Our study with *C. lipolytica* showed that urea as an additional nitrogen source in the two tested media did not cause any decrease in growth rate when batch fermentation at exponential phase was used. These results suggested a decrease in the interfacial tension of babassu oil-containing YSW-B_2 and YSW-B_3 media caused by *C. lipolytica*, presumably due to release of fatty acids in the initial stages of fermentation. The production of fatty acids declines after 60 h of fermentation, but the interfacial tension continues.

The bioemulsifiers obtained from YSW-G and YSW-B_3 media were composed by proteins (54.3 and 43.0% w/v respectively), carbohydrates (35.5 and 40.0% w/v respectively) and lipids (8.4 and 16.0% w/v respectively).

The high production of emulsifiers in both media was probably caused by the presence of potassium phosphate. Palejwala and Desai (7) observed the influence of phosphate anions in the medium on the production of emulsifiers. It should be noticed that emulsifiers were produced in the two examined media (YSW-B_2 and YSW-B_3) using low-cost substrates (babassu oil and seawater), which can be used for bioemulsifier production by *Candida lipolytica*.

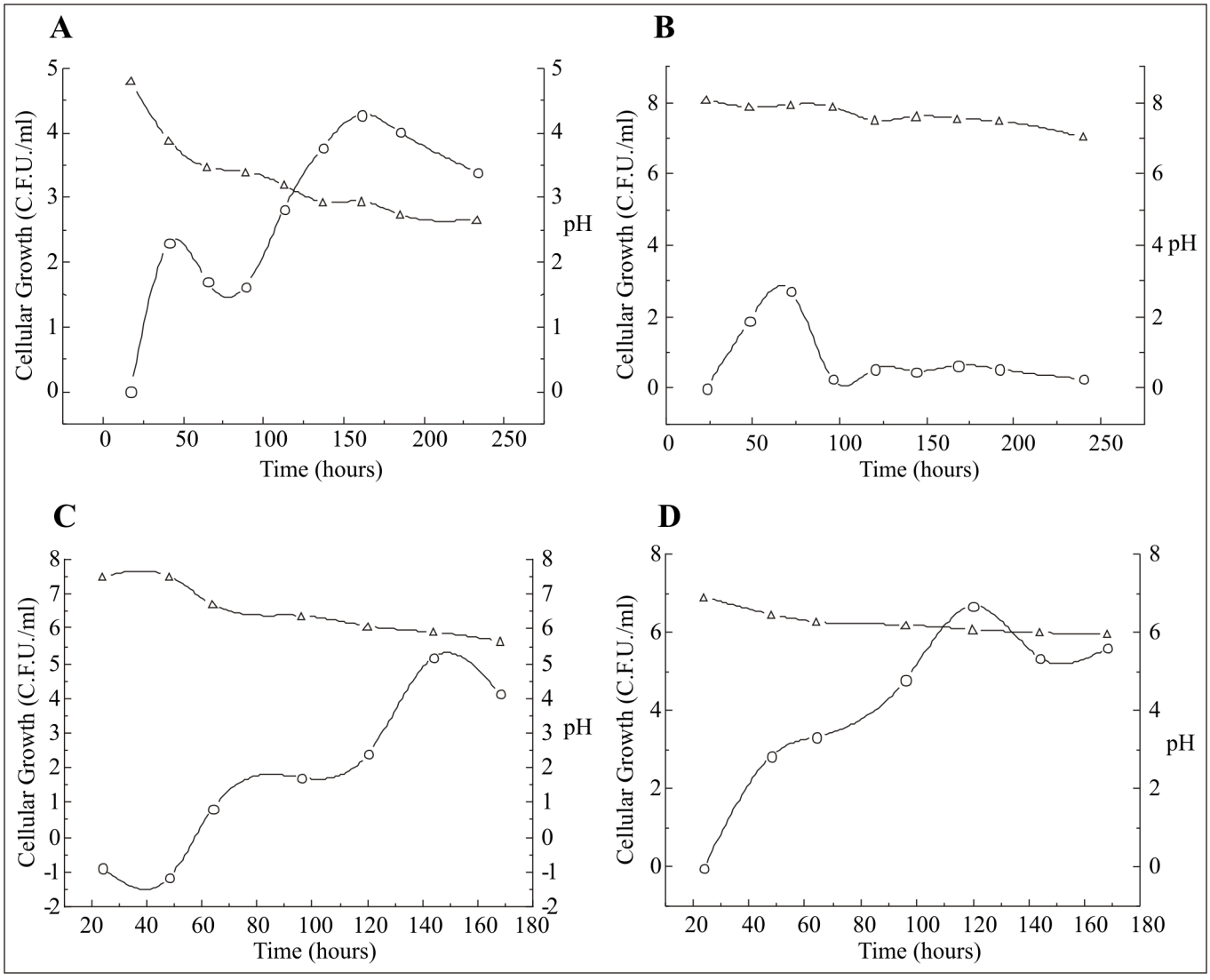

Figure 1. Growth (❍) of *Candida lipolytica* and pH (△) of YSW-G (A), YSW-G_1 (B), YSW-B_2 (C) and YSW-B_3 (D) media incubated at 28ºC for 240 hours, 150 rpm.

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RESUMO

Novos bioemulsificantes produzidos por *Candida lipolytica* usando D-glicose e óleo de babaçu como fontes de carbono

Candida lipolytica IA 1055 produziu biosurfactantes extracelulares com atividade de emulsificação, através de fermentação utilizando óleo de babaçu e D-glicose como fontes de carbono. A água do mar diluída a 50% suplementada com uréia, sulfato de amônio e fosfato foi usada como meio basal. Os melhores resultados foram atingidos com os meios YSW-B_2 (contendo uréia, sulfato de amônio e óleo de babaçu) e YSW-B_3 (contendo uréia, sulfato de amônio, fosfato e óleo de babaçu), através de fermentação em batelada alimentada com 5% de óleo de babaçu. A velocidade específica de crescimento foi de 0,02 h^{-1} e 0,04 h^{-1}; tempo de geração de 34,6 h^{-1} e 17,3 h^{-1} e atividade de emulsificação igual a 0,666 e 0,158 unidades, respectivamente. As moléculas dos novos bioemulsificantes demonstraram ser constituídas por carboidratos, proteínas e lipídeos.

Palavras-chave: Bioemulsificante; *Candida lipolytica*; óleo de babaçu, água do mar

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CAPÍTULO II

Evaluation of a Semidefined Medium for the Production of Biosurfactant by *Candida lipolytica* Using a Factorial Design

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Evaluation of a Semidefined Medium for the Production of Biosurfactant by *Candida lipolytica* Using a Factorial Design

Vance-Harrop , M.H[1].; Barros-Neto, B.[2] & Campos-Takaki, G.M[3].

[1]Doutorado em Biologia de Fungos, Universidade Federal de Pernambuco, 50670-420 Recife-Pernambuco, Brazil

[1] LAPA/Recife, Ministério da Agricultura, Pecuária e do Abastecimento.

[2] Departamento de Química Fundamental, Universidade Federal de Pernambuco, 50670-420 Recife-Pernambuco, Brazil

[3] Departamento de Química e Núcleo de Pesquisas em Ciências Ambientais,
Universidade Católica de Pernambuco, 50050-590 Recife-Pernambuco, Brazil

Abstract.

The composition of culture media for biosurfactant production in batch cultures of *Candida lipolytica* by a fractional factorial design has been studied. A set of 16 experiments with centre points in duplicate, utilizing five factors (yeast extract, urea, ammonium sulfate, potassium phosphate and babassu oil) at two levels were statistically combined. The results show that all the factors studied were found to influence biosurfactant production, and that higher biosurfactant synthesis is obtained when yeast extract, urea, and potassium phosphate are at a high level. However, with ammonium sulfate at a lower level and babassu oil, the level is independent of the response (biosurfactant production).

Keywords: Biosurfactant, Factorial design, Medium optimization, *Candida lipolytica.*

Introduction

Surfactants are those chemicals which are produced by microorganisms but whose amphipatic molecules have both clearly defined hydrophobic and hydrophilic groups. These moieties partition preferentially at the interface between fluid phases with different degrees of polarity, such as oil-water or air-water interfaces (Healy et al. 1996). These compounds have the property of reducing surface and interfacial tension in both aqueous solutions and hydrocarbon mixtures, and thereby have potential applications in the recovery of oil, and the pharmaceutical and food industries (Banat et al. 2000).

The biosurfactants have many different chemical structures. Microorganisms can produce surfactants when grown on many different substrates, ranging from carbohydrates to hydrocarbons. Changing the substrate often alters the structure of the molecule, in turn, altering its surfactant properties (Cooper, 1986). Biosurfactants have several advantages over their chemical counterparts, such as lower toxicity, higher biodegradability, better environmental compatibility and the ability to be synthesized from renewable feedstocks (Fiechter, 1992).

Currently, worldwide synthetic surfactant production exceeds three million tons per year and is expected to rise annually.

Most of the commercially available surfactants are chemical surfactants, mainly petroleum-derived. However, rapid advances in biotechnology and increased environmental awareness on the part of consumers, combined with expected new legislation in many countries, has provided further impetus for serious consideration of biological surfactants as possible alternatives to synthetic ones (Banat et al.2000). Surfactants have long been among the most versatile of process chemicals. The market for them is extremely competitive and this gives rise to a demand to expand the stock by developing new products. In this regard, biosurfactants are promising

candidates. However, the cost of biosurfactant production is about three to ten times higher than that of their chemical counterparts. Thus, future biosurfactant applications will be governed mainly by the overall economic gain of production versus application. The fermentation process holds the key to improving the overall process economics in biosurfactant production. Generally, biosurfactants are produced by growth on hydrocarbons which are usually expensive and therefore increase the overall process cost (Desai & Banat, 1997).

In the search for cheaper raw materials for production of these compounds, the use of agro-industrial by-products and industrial effluents such olive oil mill effluent and other edible vegetable oils have recently shown great promise (Mercadé et al.1993; Cooper& Paddock, 1984).

A number of attempts have been made to increase biosurfactant productivity by manipulating fermentation conditions and medium composition. (McCaffrey & Cooper 1995; Casas et al. 1997). Rao et al. (2000) performed a study to achieve high product yields by statistical optimization of medium for the production of recombinant hirudin from *Saccharomyces cerevisiae* using a response surface methodology.

The combined effects of initial xylose concentration and inoculum level on xyllitol production by *Candida guilliermondii* from rice straw hydrolysate were studied using a 2^2 full-factorial central composite design for experimental design and analysis of results (Silva and Roberto, 2001). Optimization using factorial design and response surface analysis specifically meets the requirement that optimal experimental conditions be determined.

The Ascomycetous yeast *Candida lipolytica* is currently used as a model organism for fundamental studies and also for biotechnological applications of the ability to produce surface-active molecules during growth on water-immiscible substrates (Barth & Gaillardin, 1997). During the fermentative processes, in the particular case of secondary metabolites, the interaction

between growth metabolism and product secretion is critically influenced by growth-limiting nutrient concentrations.

Experimental design techniques are very useful tools for this purpose, providing evidence of the relative influence of various factors studied and determining the optimal concentration calculated for a given target such as maximal growth or maximal metabolite production. In a previous study of biosurfactant production from *C. lipolytica*, this yeast demonstrated the ability to produce molecules with surfactant properties in culture media containing natural sea water and distilled water in the liquid phase. The solid phase used ammonium sulfate, urea, potassium phosphate and babassu oil, an edible oil used in the North region of Brazil, as a carbon source. (Vance-Harrop, et al. 2003).

The influence of some selected nutrients - babassu oil as a carbon source; ammonium sulfate, urea and potassium phosphate as a nitrogen source and the yeast extract essential for the growth - was tested using a factorial design and analysis of experiments in three stages. The purpose of this was to determine the best concentrations in a semi-defined medium for the growth and biosurfactant production by the yeast *C. lipolytica.*

MATERIALS AND METHODS

Microorganism *Candida lipolytica*- Harrison (Diddens & Lodder) IA 1055, belonging to the culture collection of the Departamento de Antibióticos - Universidade Federal de Pernambuco, was used in this study. The strain was grown on a Yeast Mold Agar (YMA) medium and was maintained at 4°C, at the Núcleo de Pesquisas em Ciências Ambientais – UNICAP (UCP 00065).

Culture Conditions The media used for the experiments consisted of the following: yeast extract, ammonium sulfate, urea, potassium phosphate, and babassu oil. Distilled water and natural seawater were added at the liquid phase (Table 1). The pH of the media was adjusted to 5.3.

The inocula were prepared in an Erlenmeyer flask containing 50 ml of Yeast Mold Broth (YMB) and were inoculated using a microbial loop, incubated in an orbital shaker at 250 rpm and 28°C for 24h. All fermentations were conducted in Erlenmeyer flasks with a capacity of 1000 ml containing 300 ml of growth media (Table 1). Immediately after the inoculation of 5% of 10^7 cells/ml, the flasks were incubated for 96 hours at 28°C in an orbital shaker at 150 rpm. The growth profile was accompanied by biomass and pH determination, and emulsification activity.

Assay of emulsification activity. Emulsification activity was determined using the method developed by Cirigliano & Carman, 1984. An aliquot of cultures was filter sterilized using a Millipore 0.22μm membrane filter. This sample (2 ml) was diluted with 2 ml of 0.1 M sodium acetate buffer (pH 3.0) and 1 ml of n-hexadecane was added. The mixture was shaken in a vortex for 2 minutes and allowed to stand for 10 min. The turbidity (measure of oil dispersion) was

measured spectrophotometrically at 540 nm. The absorbance was multiplied by the dilution and expressed as emulsification activity (AE).

Determination of the dry weight of the culture Culture samples (10 ml) were filtered through previously weighed nitrocellulose filters (0.22 μm-pore-size). After removal of the medium, the filters were washed with demineralized water, dried in an oven at 70°C for 24 h, cooled in a desiccator and weighed. They were then repeatedly re-weighed until a constant dry weight was obtained. Duplicate determinations gave results that varied by < 1%.

pH measurement Samples were taken from the cultures when fermentation had ceased. When the samples had been brought to a uniform temperature of 25°C, the pH of the supernatants was measured using an Orion, model 310, pHmeter.

Factorial design The factorial design used was developed in the course of three assays. Factorial design 1- a $2^{(5-1)}$ fractional factorial design based on five experimental factors at two levels, augmented with a center point run in quadruplicate, was used to evaluate culture conditions for biosurfactant production (the variable response). The real and coded values of the factors involved in this design are shown in Tables 1 and 2, respectively. Factorial design 2 - a full 2^4 factorial design based on four of the five variables in Table 1 was used for a follow-up study. The level combinations for this design are given in Table 3. Finally, factorial design 3 - a full 2^3 factorial with centre points in duplicate was performed to assess the effect of a lack of urea on the culture medium (Table 4).

Statistical analysis All data were analyzed using the Statistica software package (StatSoft Inc., 1996). The statistical significance of the results was established at the $p< 0.05$ level.

RESULTS AND DISCUSSION

The yeast *C. lipolytica* produces biosurfactants when it grows in water-insoluble substrates (Vance-Harrop et al. 2003). An experimental design was used to determine the best concentrations of the media components that have previously been used. This study was accomplished in three consecutive steps: first, a fractional factorial design in a set of 16 experiments with two center points, in duplicate, was performed to determine the variables that could affect biosurfactant production. Then a 2^4 design was performed to evaluate medium performance in the absence of the nitrogen source, ammonium sulfate, which showed a negative effect on production, in the initial analysis. Finally, a 2^3 design was performed under the same conditions, but with the further exclusion of urea, to evaluate the effect of the remaining three factors on biosurfactant production.

In the first (2^{5-1}) design, the best responses were obtained at runs 8, 10, 13, 14, with 5.060, 4.950, 4.932 and 4.844 of absorbance at 540nm, respectively (Figure 1). The contrasts associated with the main effects of the five factors (yeast extract, urea, sulfate, phosphate and babassu oil) were calculated, and the main effects of phosphate, yeast extract, urea and babassu oil were all found to be positive, while that of ammonium sulfate proved negative, as shown in the Pareto plot in Figure 2. Thus, an interesting conclusion of the first part of this study was that for the best conditions for biosurfactant production yeast extract, phosphate, and urea should be used at +1 levels and ammonium at the -1 level (Table 5). The most significant effects were found with the water-insoluble carbon source, and the phosphate, linked by the energy production routes of the cell metabolism (Casas et al. 1997). Indeed, the carbon source is very important in the

biosurfactant production process, especially the immiscible ones (Cooper and Paddock, 1984). The importance of phosphate in the same process is also related by Reisfeld et al.(1972), suggesting that more rapid dispersion of oil in seawater took place in buffered sea water, catalyzed by phosphate.

A 2^4 factorial design, excluding ammonium sulfate, was subsequently carried out to determine the influence of the other four factors: yeast extract, urea, potassium phosphate and babassu oil, at the same levels. The responses obtained using this design are plotted in Figure 3. Babassu oil and yeast extract were found to have significant ($P<0.05$) effects on the response. The best conditions for biosurfactant production were those of run 12 (Figure 3). Among the four variables investigated, the factors babassu oil and yeast extract had the most important effect on biosurfactant production (Figure 4). Babassu oil has previously furnished good results in this process, and yeast extract was found to be essential for cell growth. The analysis shows that significant interactions between factors occurred for babassu oil x yeast extract, yeast extract x phosphate and yeast extract x urea (Figure 5), basically replicating the conclusions reached using the first design.

In the final 2^3 design, the most significant biosurfactant production occurred in run 8, as indicated by the high absorbance, the parameter used to detect the emulsification activity (AE) (Figure 6). The significance of the carbon source is shown in Figure 7. The only nitrogen source in this experiment is yeast extract, which contains organic growth nutrients to supply some vitamins, Scioli and Vollaro, (1997), and mineral salts contained in sea water, an ingredient of the liquid phase medium. A positive interaction between phosphate and babassu oil can also be seen in Figure 7. The other interactions do not appear to be statistically significant. In the two last

designs, the biomass and pH were also determined at the end of fermentation (Table 3 and 4). The economics of the production of biosurfactants must be worked out, if they are to compete with their chemical counterparts. The choice of inexpensive and renewable raw materials is important for the overall economic viability of the process, because these account for fifty percent of the final production cost (Zhou and Kosaric, 1995). In our study, babassu oil was used as an inexpensive and plentiful source of carbon for biosurfactant production. Babassu oil is of edible standard, does not contain preservative oils, and is used for cooking in the North region of Brazil. The relationship between metabolite production and the growth of the strain producer has been observed by Leal-Sánchez et al. (2002). They have shown that there is no clear-cut correlation between maximal Bacteriocin production and the numbers of *Lactobacillus plantarum* LPCO10 cells at the same points. These results are similar to ours. This may have been because the nutrients were consumed by cells for biosurfactant biosynthesis rather than for the growth. On the other hand, Kimmel et al. (1998) showed in their results that a profile of the fermentation conducted under optimal conditions for exopolysaccharide (EPS) production was growth-associated. Growth associated with the production does not occur with some other EPS-producing strains, such as *Xanthomonas* and *Alcaligenes spp*. (Sutherland, 1990). As expected, the capacity to sustain the large fluxes of carbon and energy required for rapid metabolite production appears to be inversely related to the growth efficiency of microorganisms, principally in the case of metabolites like biosurfactants, which are composed of moieties with significantly different oxidation states (Linton, 1991). This observation has been previously related by Rosenberg et al. (1979), whose fermentation studies using oils as a carbon source demonstrated that growth rate can be limited by the interfacial surface area between water and oil. Other studies

confirm that biosurfactant production is related to slowly growing cells (Guerra-Santos et al.1984; Reiling et al.1986).

In conclusion, these results show that all the factors studied (yeast extract, urea, ammonium sulfate, potassium phosphate and babassu oil) appear to influence biosurfactant production, and higher biosurfactant synthesis is obtained when yeast extract, urea, potassium phosphate are at higher levels, while ammonium sulfate is at a lower level. For babassu oil, the level selected is of no consequence for the range studied here. This indicates that the combined effect of all the independent variables significantly contributes to the enhancement of biosurfactant production. These conclusions are interesting from an economic point of view, since with high substrate concentrations it is possible to optimize biosurfactant production. Based on the experimental results it can be concluded that *Candida lipolytica* is able to use babassu oil as an insoluble carbon source as a substrate for producing biosurfactant. Therefore, it is feasible to use relatively inexpensive and renewable substrates for biosurfactant production.

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Table 1. Concentration levels of surfactant culture media for surfactant production.

Compound	Concentration (g/100ml) -1	(g/100ml) +1
Yeast extract	0,1	1
Ammonium sulfate	0,1	1
Urea	0,25	0,5
Potassium phosphate	0,1	1
Babassu oil	5	7,5

Table 2. Fractional factorial 2^{5-1} design (in the coded values specified in Table 1) used to evaluate biosurfactant production by *Candida lipolytica*.

	Factor level					Response[a]
Run	Yeast extract	Ammonium	Urea	Phosphate	Babassu	AE (540nm)
1	-1	-1	-1	-1	+1	4.804
2	+1	-1	-1	-1	-1	4.044
3	-1	+1	-1	-1	-1	4.056
4	+1	+1	-1	-1	+1	4.142
5	-1	-1	+1	-1	-1	4.034
6	+1	-1	+1	-1	+1	4.732
7	-1	+1	+1	-1	+1	4.344
8	+1	+1	+1	-1	-1	5.060
9	-1	-1	-1	+1	-1	4.584
10	+1	-1	-1	+1	+1	4.950
11	-1	+1	-1	+1	+1	4.280
12	+1	+1	-1	+1	-1	4.576
13	-1	-1	+1	+1	+1	4.932
14	+1	-1	+1	+1	-1	4.844
15	-1	+1	+1	+1	-1	4.240
16	+1	+1	+1	+1	+1	4.580
17 (C)	0	0	0	0	0	4.530
17 (C)	0	0	0	0	0	4.532
17 (C)	0	0	0	0	0	4.500
17 (C)	0	0	0	0	0	4.438

[a] Responses obtained with each run are shown as Emulsification Activity (AE) estimated at an absorbance rate of 540 nm.

Table 3. Full 2^4 follow-up design, excluding ammonium sulfate from the culture media for biosurfactant production. Coding as given in Table 1

	Factor level				Response[a]		
Run	Yeast extract	Urea	Phosphate	Babassu	AE (540nm)	Biomass[b]	pH
1	-1	-1	-1	-1	1.476	4.81	5.51
2	+1	-1	-1	-1	1.728	6.16	5.52
3	-1	+1	-1	-1	4.716	6.13	5.49
4	+1	+1	-1	-1	3.120	5.09	5.59
5	-1	-1	+1	-1	2.270	8.94	5.48
6	+1	-1	+1	-1	1.872	7.96	5.61
7	-1	+1	+1	-1	1.860	4.60	5.68
8	+1	+1	+1	-1	4.884	7.55	5.79
9	-1	-1	-1	+1	2.264	5.69	5.36
10	+1	-1	-1	+1	4.528	6.07	5.36
11	-1	+1	-1	+1	4.700	4.83	5.72
12	+1	+1	-1	+1	5.180	6.21	5.53
13	-1	-1	+1	+1	4.636	6.16	5.49
14	+1	-1	+1	+1	4.802	7.13	5.47
15	-1	+1	+1	+1	1.286	6.86	5.82
16	+1	+1	+1	+1	4.986	5.72	5.65
17 (C)	0	0	0	0	3.688	5.63	5.62
17 (C)	0	0	0	0	4.374	4.98	5.47
17 (C)	0	0	0	0	4.652	7.43	5.56
17 (C)	0	0	0	0	5.020	4.29	5.59

[a] Responses obtained with each run are shown as emulsification activity (AE) estimated at an absorbance rate of 540 nm.

[b] Biomass is expressed in g/liter.

Table 4. Full 2^3 factorial design, excluding ammonium sulfate and urea.

Coding as given in table 1.

	Factor level			Response[a]		
Run	Yeast extract	Phosphate	Babassu	AE (540 nm)	Biomass[b]	pH
1	-1	-1	-1	3.744	4.00	5.28
2	+1	-1	-1	4.250	4.51	5.12
3	-1	+1	-1	3.720	2.96	5.19
4	+1	+1	-1	3.220	8.21	5.37
5	-1	-1	+1	3.512	2.98	4.90
6	+1	-1	+1	4.484	3.34	4.99
7	-1	+1	+1	4.120	6.91	5.30
8	-1	+1	+1	4.822	3.1	5.14
9 (C)	0	0	0	4.418	5.1	5.22
9 (C)	0	0	0	4.714	6.26	5.24
9 (C)	0	0	0	4.230	4.6	5.25
9 (C)	0	0	0	4.376	5.29	5.28

[a] Response surface of biosurfactant production by *Candida lipolytica* in emulsification activity (AE) estimated at an absorbance rate of 540nm.

[b] Biomass determination is expressed in g/l^{-1}

Table 5. Model fitting results for production of biosurfactants by *Candida lipolytica*.

Effect Estimates; Var.:ABSORVAN; R-sqr=.99693; Adj:.98058
2**(5-1) design; MS Pure Error=.0019227
DV: ABSORVAN

		Std.Err.			-95.%	+95.%		Std.Err.	-95.%	+95.%
	Effect	Pure Err	t(3)	p	Cnf.Limt	Cnf.Limt	Coeff.	Coeff.	Cnf.Limt	Cnf.Limt
Mean/Interc.	4.512625	.010962	411.6586	.000000	4.477739	4.547511	4.512625	.010962	4.477739	4.547511
Curvatr.	-.025250	.049024	-.5151	.642061	-.181266	.130766	-.012625	.024512	-.090633	.065383
(1)YEAST EXT.	.206750	.021924	9.4303	.002527	.136978	.276522	.103375	.010962	.068489	.138261
(2)AMMONIUM	-.205750	.021924	-9.3846	.002563	-.275522	-.135978	-.102875	.010962	-.137761	-.067989
(3)UREA	.166250	.021924	7.5830	.004758	.096478	.236022	.083125	.010962	.048239	.118011
(4)PHOSPHATE	.221250	.021924	10.0916	.002072	.151478	.291022	.110625	.010962	.075739	.145511
(5)BABASSU	.165750	.021924	7.5602	.004799	.095978	.235522	.082875	.010962	.047989	.117761
1 by 2	.152750	.021924	6.9672	.006067	.082978	.222522	.076375	.010962	.041489	.111261
1 by 3	.209750	.021924	9.5671	.002423	.139978	.279522	.104875	.010962	.069989	.139761
1 by 4	.021750	.021924	.9921	.394299	-.048022	.091522	.010875	.010962	-.024011	.045761
1 by 5	-.195750	.021924	-8.9285	.002964	-.265522	-.125978	-.097875	.010962	-.132761	-.062989
2 by 3	.126250	.021924	5.7585	.010406	.056478	.196022	.063125	.010962	.028239	.098011
2 by 4	-.202750	.021924	-9.2478	.002675	-.272522	-.132978	-.101375	.010962	-.136261	-.066489
2 by 5	-.312250	.021924	-14.2423	.000750	-.382022	-.242478	-.156125	.010962	-.191011	-.121239
3 by 4	-.114750	.021924	-5.2340	.013572	-.184522	-.044978	-.057375	.010962	-.092261	-.022489
3 by 5	-.063250	.021924	-2.8850	.063273	-.133022	.006522	-.031625	.010962	-.066511	.003261
4 by 5	-.041250	.021924	-1.8815	.156464	-.111022	.028522	-.020625	.010962	-.055511	.014261

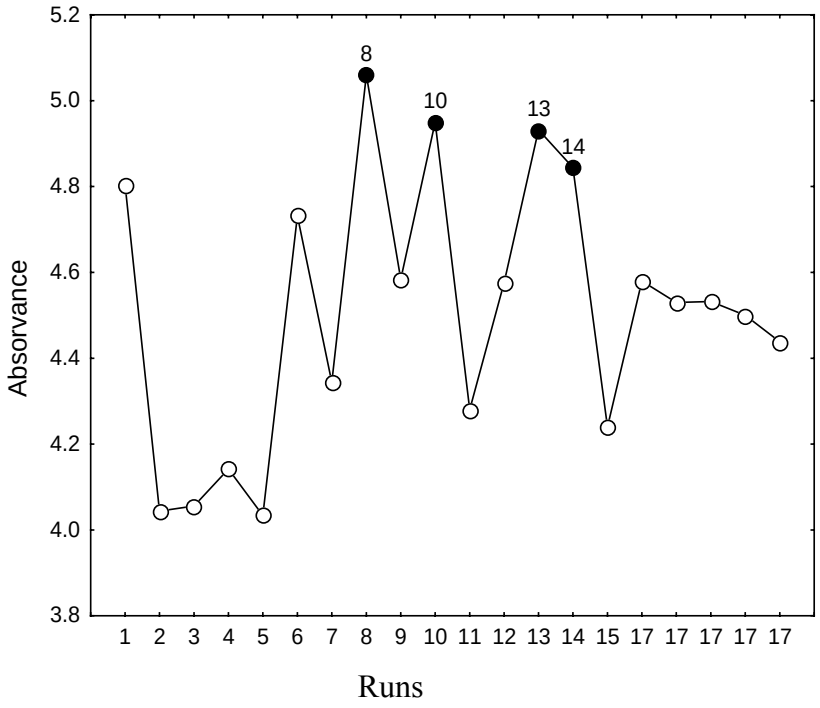

Figure 1. Response surface of biosurfactant production by *Candida lipolytica* in emulsification activity (AE) estimated at an absorbance rate of 540 nm for the $2^{(5-1)}$ design experiments. Symbol: ● shows the runs that obtained the high emulsification activity (AE).

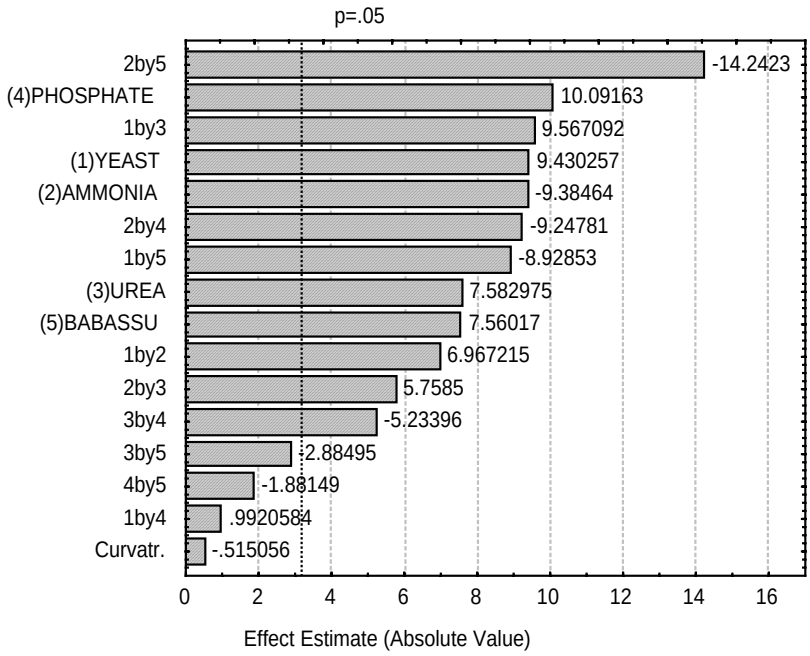

Figure 2. Pareto chart of standardized estimated effects for the five factors tested in the first design for biosurfactant production by *Candida lipolytica*. The point at which the effect estimates were statistically significant (at P= 0.05) is indicated by the vertical solid line.

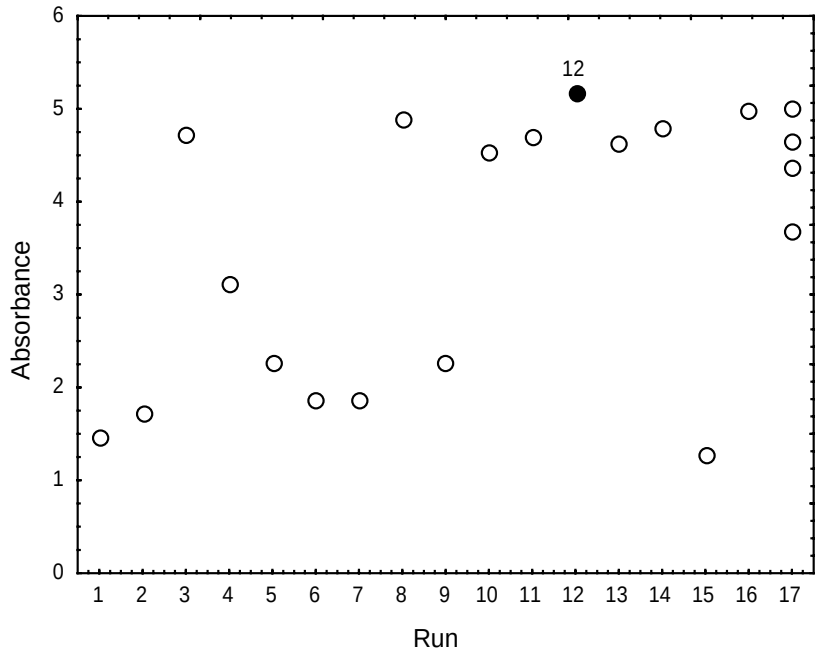

Figure 3. Responses for the full 2^4 design of experiments, estimated at an absorbance rate of 540 nm, for emulsification activity (AE). The symbol: ● shows run 12 as the high AE.

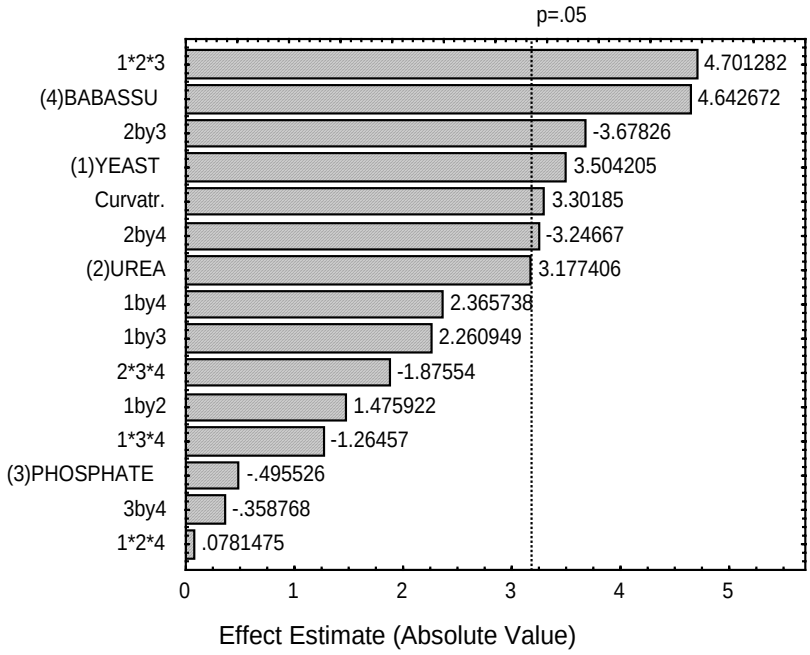

Figure 4. Pareto chart of the effects calculated form the responses in Figure 3 of the full factorial design (2^4) for biosurfactant production by *Candida lipolytica.* The point at which the effect estimates were statistically significant (at P=0.05) is indicated by the vertical solid line.

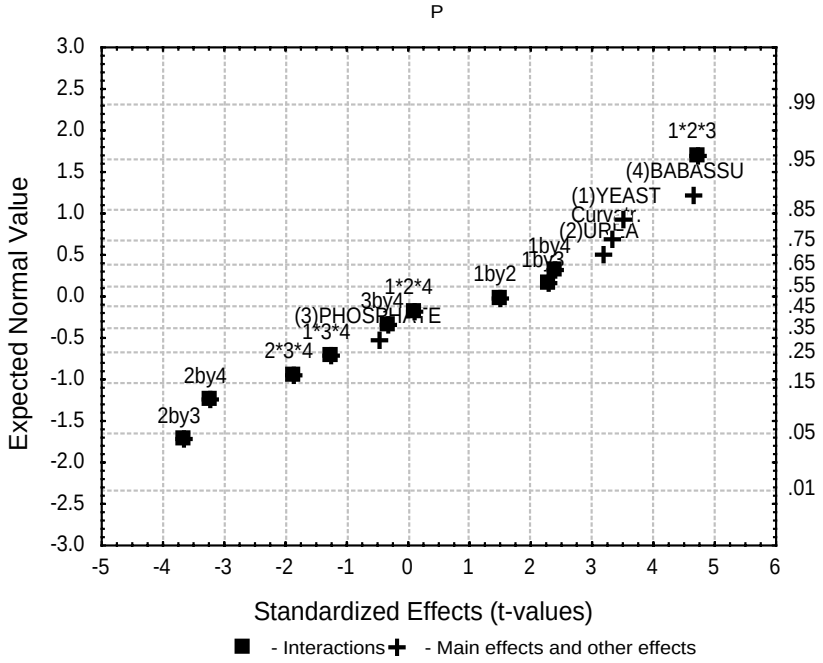

Figure 5. Normal probability plot of the effects calculated for the full 2^4 design of experiments for biosurfactant production by *Candida lipolytica.*

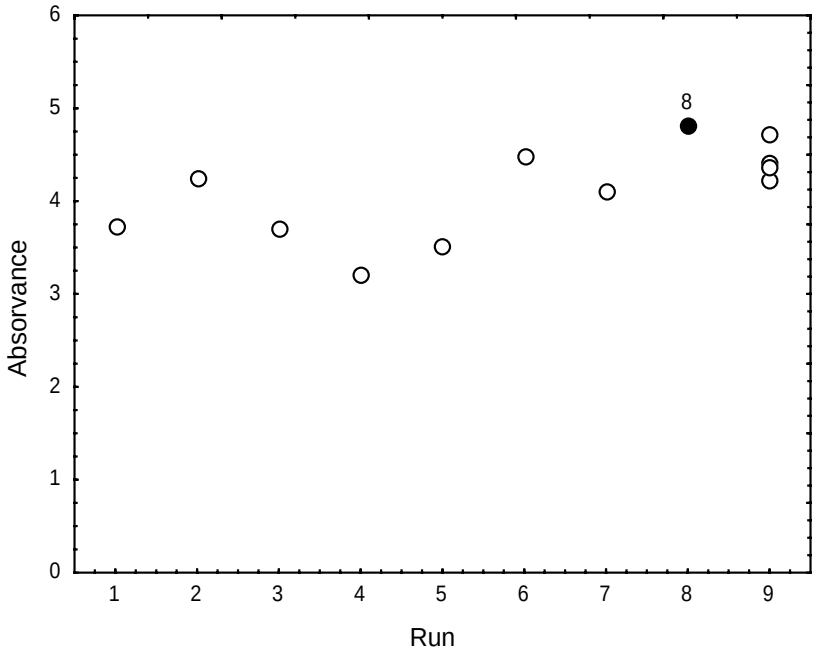

Figure 6. Responses for the full 2^3 design of experiments, for the biosurfactant production by *Candida lipolytica* estimated at an absorbance rate of 540 nm for emulsification activity (AE). Symbol: ● shows run 8 as the high AE.

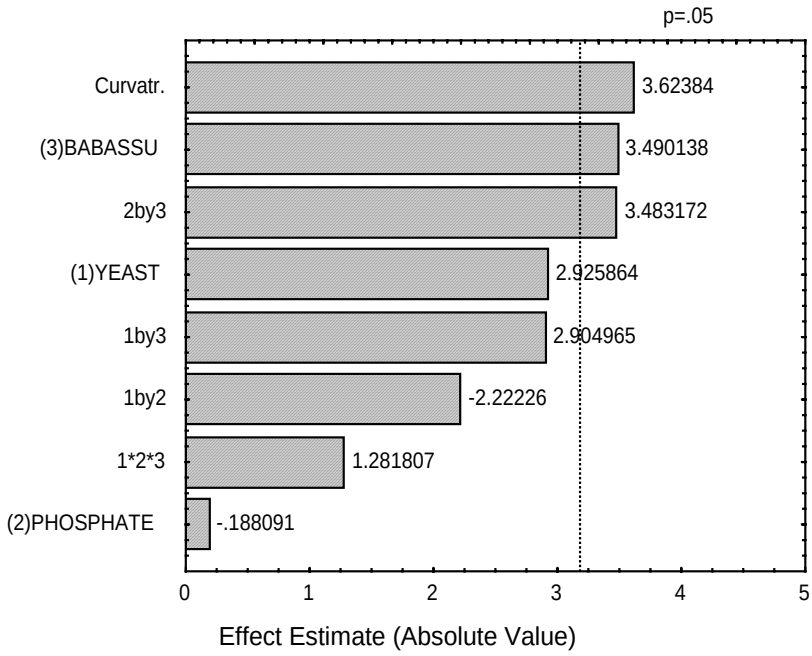

Figure 7. Pareto chart of the effects calculated from the responses in Figure 6, of a full 2^3 design for biosurfactant production by *Candida lipolytica* .The point at which the effect estimates were statistically significant (at p= 0.05) is indicated by the vertical solid line.

CAPÍTULO III

Remotion of Pyrene by *Candida lipolytica* under Mixed Substrates Conditions

Manuscrito submetido ao:
FEMS Yeast Research

Remotion of Pyrene by *Candida lipolytica* under Mixed Substrates Conditions

Vance-Harrop, M.V [1]; Shiosaki, R. K [2]. & Campos-Takaki, G. M [3].

[1] Doutorado em Biologia de Fungos, Universidade Federal de Pernambuco, 50670-420 Recife-Pernambuco, Brasil

[1] LAPA/Recife, Ministério da Agricultura,Pecuária e do Abastecimento.

[2] Doutorado em Ciências Biológicas, Universidade Federal de Pernambuco, 50670-420 Recife-Pernambuco, Brasil

[3] Departamento de Química e Núcleo de Pesquisas em Ciências Ambientais,
Universidade Católica de Pernambuco, 50050-590 Recife-Pernambuco, Brasil

Abstract

Candida lipolytica has shown an organism suitable for the biosurfactant production and degradation of hydrophobic substrates. Alternative treatments were carried out using *Candida lipolytica* to degradate the polycyclic aromatic hydrocarbon pyrene, derivative from petroleum, using five different culture media. The mixed substrates consisted of glucose and sucrose as soluble carbon sources and babassu oil and pyrene as insoluble sources. The results suggested that biosorption and co-metabolism occurred during the *Candida lipolytica* growth in substrate interactions in the mixture of the sources. The remotion procedures consisted of cultivation in sucrose, glucose and babassu oil substrates, added of 10 mgl^{-1} of pyrene during 5 days. The results demonstrated a high removal rate of pyrene in media I and IV with 99.02% and 97.51% respectively.

Keywords: *Candida lipolytica*, Biosorption, Remotion, Pyrene.

1. Introduction

Polycyclic aromatic hydrocarbon (PAHs) are ubiquitous persistent environmental contaminants, occur as common constituents of petroleum, coal tar and as by-products of industrial activities. PAHs are of concern because some of them are known or suspected carcinogens and mutagens and exposure to them may be represent a significant risc to human health populations [1]. Their physical-chemical properties, which include hidrophobicity and high adsorption coefficient, combine to make soils and sediments environmental sinks for PAHs [2]. The oil industry is responsible for the generation if high amounts of oily residues. In Brazil, it is estimated that approximately 1% of the total oil processed in a refinery is discarded as oily sludge [3].

Pyrene, a PAH with four fused aromatic rings, has been detected in environmental samples and used as indicator for PAHs-contaminated wastes monitoring [4].

Bioremediation technologies have increasingly been proposed to decontaminate those sites polluted by PAHs [5]. However, polluted sites with PAHs frequently resist a fast and complete clean up. For an efficient remediation process it is important that the microorganisms involved have a potential degradation pathway so that no toxic degradation products accumulate in soil [6]. Biological processes have been received special attention, principally because is an economical and environmentally attractive solution for removing toxic compounds from contaminated sites [7].

Microbial degradation is a major factor affecting the persistence of PAHs in environment [8]. Several known bacterial PAHs degraders have been mediated by *Pseudomonas* [9,10]; *Mycobacterium* [8,11]. The ability of fungi to transform a wide variety of hazardous chemicals have aroused interest in using them in bioremediation process [12]. Among other fungi used in bioremediation, the yeasts are important in cleaning industrial effluents of unwanted chemicals.

A study related with nine yeasts strains, *Candida lipolytica* showed the highest ability for remove toxic dyes [13]. Co-metabolism process has recently emerged as an important technique for the biotreatment of many recalcitrant compounds [14]. Co-metabolism is the fortuitous biotransformation of a non-growth-supporting compound by a microorganism. The biotransformation process is catalysed by a non-specific enzyme or cofactor. Co-metabolism also requires that these transformations be concurrent with the metabolism of a growth-supporting substrate or another transformable compound [15].

In this work, we investigated the abilities of *Candida lipolytica* to co-metabolize the hydrocarbon pyrene at low concentration (10 mgl^{-1}). It is observed that this yeast readily degraded pyrene. The residual concentration of PAH was measured and the effects of growth substrates on yeast co-metabolism were described.

2. Materials and Methods

2. 1. Microorganism *Candida lipolytica* strain UCP 00065 used in this work was obtained at culture collection, Universidade Católica de Pernambuco. During the present investigation this organism was routinely maintained at 4^0C on Yeast Mold Agar (YMA) slants [16].

2. 2. Chemicals Pyrene hydrocarbon was obtained from Wako Pure Chemical Industries, Ltd. All chemicals were analytical grade. All organic solvents were glass-distilled grade or HPLC grade.

2. 3. Media and culture conditions

The inoculum for each experiment was prepared in Yeast Mold Broth (YMB). All cultivations were performed with the mineral salts medium previously described [17] and enhanced by a factorial design, by these authors (to be submmited) and modified for a remotion of pyrene experiments, by supplementation with desired carbon sources: soluble- glucose and sucrose and insolubles- pyrene and babassu oil (Table 1).

Candida lipolytica was grown in the media as indicated in Table 1; the pH were adjusted to 5.3 and distributed 300 ml by flasks of 1000 ml of capacity and autoclaved at 121^0C for 20 min. The babassu oil and pyrene were added directly from stock solutions to give the final concentrations of 62.5ml l^{-1} and 10 mgl^{-1} respectively. Controls flasks as abiotic conditions were used.

The flasks were incubated in dark conditions on a rotary shaker at 150 rpm and 28^0 C for 5 days. Aliquots were collected for growth profile and pH measurements. Growth of organisms in the media was monitored by viable cells enumeration as CFU by quintuplicate spread plating of serially diluted samples on YMA. Plates were incubated at 28^0 C for approximately 5 days prior to colony counting. The pH was measured using a pH meter Orion, model 310.

2. 4. Estimation of kinetics parameters Specific rate coefficients, (μ) were estimated by log transformed CFU-versus-time profiles.

2. 5. Analytical methods

2. 5. 1. Measurement of pyrene remotion The pyrene remotion was accompanied by monitoring the disappearance of pyrene in the media versus abiotic controls**.**

2. 5. 2. Chromatography evaluation After 5 days of incubation, the cultures were centrifuged to remove cells and the supernatants were extracted three times with ethyl acetate (1:1, v/v). The extracts were dried over anhydrous sodium sulfate and evaporated to partial dryness with a rotary evaporator. After dissolved in acetone the extracts were applied to Thin Layer Chromatograph plates (Silica gel plates $60F_{254}$ Merck). The plates were developed in benzene and then exposed to UV light. Bands that fluoresced strongly under UV light (254nm) were scraped off the plates, redissolved in acetone, and filtered through a Millipore 0,02μm-pore-size filter. The filtrates were evaporated and the residue was dissolved in acetonitrile and analysed by High Performance Liquid Chromathography (HPLC).

2. 5. 3. High Performance Liquid Chromathography Reverse-phase HPLC was performed on a Varian liquid chromatograph system comprising a solvent delivery system, and a variable-wavelength UV-visible light detector; and this system was controlled by Varian Star chromatographic software (version 4.01). Acetone-dissolved extracts of tests and controls were appropriately diluted and injected onto a C_{18} column. The mobile phase consisted by acetonitrile and the flow rate was 1.0 ml $min^{-1.}$ Compounds in the eluate were detected at 254 nm. The results are expressed as the percentage of pyrene remaining relative to abiotic controls after 5 days of incubation.

3. Results

3. 1. Growth on liquid media

In previous work, media for surfactant production by *Candida lipolytica* were evaluated. The best and economic medium was choose in a factorial design study performed and this medium was modified to observed the growth and ability to remove pyrene at concentration of 10 mgl^{-1} by yeast *Candida lipolytica* UCP 00065. The medium which consisted of yeast extract 5,5 gl^{-1}, urea 3,75 gl^{-1}, and potassium phosphate 5,5 gl^{-1}, was used as the basal medium. Five different media have been used in order to obtain the most efficient conditions in substrate interactions involving co-metabolism of this recalcitrant compound (Table1). *Candida lipolytica* has showed ability to degrade water immiscible compounds like edible oils and surfactants production [17] and was the best conditions of degradated crude oil [18]. The babassu oil is a food grade without preservatives utilized in culinary of the north region of Brazil and it is a substrate inexpensive and plentiful for use as insoluble carbon source.

In the medium I, the basal medium was supplemented with babassu oil as carbon source, added of 10 mgl^{-1} of pyrene (Table 1), the yeast showed an inhibition of growth, by a killing curve (Fig. 1). With these substrate there was no microbial growth over the course of the transformation, that decaying over time. The exponential 'death' rate was not calculated.

When growth in medium II with glucose as a primary substrate and babassu oil as carbon sources and pyrene the non-growth substrate, the yeast showed normal batch kinetics and attained a cell density of approximately $1.8x10^{8}$ $cells/ml^{-1}$ at 72 hours of fermentation. Microbial growth on 10 mg l^{-1} of pyrene had specific growth rate (μ) of 0.051 h^{-1} and generation time (g) of 13.5 hours. The cell growth and pH profile of the yeast in medium II utilizing glucose together with babassu oil on pyrene removal were shown in (Fig 2).

Figure 3 demonstrated the growth curve of *Candida lipolytica* utilizing sucrose and babassu oil as carbon sources in pyrene removal in medium III. In this experiment, two distinct exponential growth phases were clearly observed. During the first exponential growth period when sucrose was utilized the medium had specific growth rate (μ) of 0.04 h^{-1} and generation time (g) of 17.32 hours. In the second exponential phase growth the values were μ = 0.014 h^{-1} and generation time (g) of 49.5 hours. The pH of the medium increased to 7.3 after 24 hours and was decreasing at the end to 6.8 (Fig. 3).

In medium IV, added of glucose and babassu oil and non-growth substrate pyrene. The cells numbers were decreasing from the inicial period of fermentation until 72h indicated that lag phase increased. After this time, the number of viable cells increased during a 24 h period after that decreased at the end of experiment (Fig. 4).

In this growth period was observed a specific growth rate of μ= 0.032 h^{-1} and generation time (g) of 21.5 hours. The growth profiles show a lag phase (defined as the time during which the cells numbers does not increase) of several days.

The medium V had been supplemented with sucrose, babassu oil and pyrene (Table 1). After the inoculation of experiments the growth of *Candida lipolytica* demonstrated a decrease in cells viable numbers until 72 hours like medium IV. After this time, the cell growth increased until at the end of fermentation (5 days). The specific growth rate μ = 0.048 h^{-1} and generation time (g) of 14.4 hours (Fig. 5).

3. 2. Remotion of pyrene in liquid culture

The pyrene remotion rate at the end of the linear-decrease of growth curve was 99.02% for the medium I, this rate was the best attained in this work. In medium II the rate of pyrene removed was 95.78%, after the 5 days of incubation. The pyrene remotion rate at the of fermentation of medium III indicated a percentage of 91.78 of remotion in supernatant of the culture. In media IV and V the percentage of pyrene remaining in liquid culture were 2.49 and 3.13 and the pyrene remotion were 97.51 and 96.87, respectively (Table 2).

4. Discussion

During the growth in medium I was observed the highest rate of pyrene remotion in comparison with the others media. The percentage of pyrene remotion was 99.02 in a exponential 'death' curve (Fig. 1), indicating that resting cells of the yeast promoted adsorption of the hydrocarbon onto cell surface. Cells walls of yeasts contain polysaccharides as basic building blocks which have ion exchange properties, and also proteins and lipids that can accentuate the biosorption process by proffering active sites of functional groups to binding pyrene molecules [13]. In this medium the non-growth substrate (pyrene) itself exerts an inhibitory effect on this own transformation in the presence of the growth substrate (babassu oil).

Thus, it has been reported that the immediate 4-chlorophenol (4-CP) by phenol-assimilating resting cells of yeast *Candida maltosa* and ascribed it to absorption onto cell surface [19]. However, the exact reason(s) is unclear. The ability of yeast biomass to remove and accumulate heavy metals [20], and reactive dyes that are typically azo-based chromophores, has been recognize. Batch studies were conduced with different kinds of yeasts, and *Candida lipolytica* exhibited the highest dye uptake capacity, indicating the adsorptive properties of this microorganism [13]. Nevertheless, adsorption of hazardous organic compounds by

microorganisms has been rarely studied, the results obtained in a work [21], showed that dead biomass of microorganisms could remove 1,2,3,4-tetrachlorodibenzo-p-dioxin and polychlorinated dibenzofurans molecules from the medium more effectively than live cells. The biosorption of various hazards organic pollutans by inactive biomass of *Rhizopus arrhizus* has been studied [22]. Our results are similar to obtained by others authors [23], that observed in a kinetic study of phenanthrene that rapid decrease of phenanthrene concentration in medium was due to its assimilation by the cells or its adsorption to the cell wall. It was further confirmed that the assimilated or adsorbed phenanthrene was completely utilized by the *Pseudomonas mendocina* cells after 60 h of incubation. In the same study, the microbial growth on 20mgl^{-1} had a specific growth rate (μ) of 0.038 h^{-1} and a generation time of 18.3 hours, in a mineral basal medium supplemented with phenanthrene as carbon sources. This results shows a similar numbers of growth kinetics of medium III, in this first phase of exponential growth (Fig. 2). In the experiment with medium II with glucose as a primary substrate and babassu oil as carbon sources and pyrene the non-growth substrate, the yeast removed 95.78% of pyrene (Table 2). In this conditions the yeast shows a higher cell growth in a minor generation time because used the two carbon sources. In this medium the pyrene a toxic compound do not show inhibition effects in yeast growth behavior, but exhibit a prolonged exponential phase. We attributed this effect to the toxicity of non-growth substrate or its transformation products, that may be resulted in the injury of some cellular components. These observations were corroborated in intensive studies of co-metabolism of trichloroethylene (TCE). It has been found that co-metabolism of TCE lead to cells inativation, but the oxidation of growth substrate can facilitate the recovery from cell inativation by providing energy for 'de novo' protein synthesis [15].

In the medium III, *Candida lipolytica* grows utilizing sucrose and babassu oil as carbon sources soluble and insoluble, respectively. Here, in this experiment, two distinct exponential growth phases were clearly observed. During the first exponential growth period when sucrose was utilized the yeast growth shows a specific growth rate (μ) higher than the second phase, and a minor generation time (g). In contrast, the second exponential phase growth the values of (μ) were decrease and generation time (g) of 49,5 hours. The results of this batch culture shows that the presence of pyrene has a negative impact over the growth of the yeast, the lag phase increased and the growth rate decrease. This was probably due to adaptation of the insoluble substrates babassu oil and pyrene remotion and enzymatic synthesis. As demonstrated in Fig. 3, the pH medium increased to 7.3 after 24 hours and was decreasing at the end of fermentation to 6.8. The remotion rate of pyrene was 91.78%. Here, we observed that glucose as soluble carbon source used in medium II demonstrated more efficiency in pyrene remotion than sucrose, used in medium III. In a study on co-metabolism of 4-chlorophenol (4-cp) by *Pseudomonas putida* ATCC 49451 in a presence of phenol and a conventional carbon source, sodium glutamate, was demonstrated a new biphasic cell growth pattern characterized by two exponential growth phases separately by an intermediated lag phase [14]. The authors believes that this biphasic growth pattern results from the toxicity disparity of 4-chlorophenol-degrading enzyme and sodium glutamate-degrading enzymes activities. They concluded that this growth processes involves inhibition among substrates, inactivation of the cells due to the toxicity of 4-cp, and the recoverability of the cells in response to inactivation. Based on this, we ascribed that the prolongated lag phase exhibited by the medium IV was probably similar mechanisms of degradation of insoluble substrates, babassu oil and pyrene.

A set of experiments on the toxicity of polycyclic aromatic hydrocarbons on microorganisms demonstrated that amounts of naphthalene, 2-methylnaphthalene, pyrene and others result in an increased lag phase and lowered growth rate of two marine bacteria growing on these compounds confirming our results [24]. However, the polycyclic aromatic hydrocarbons are expected to have a specific toxic effects on cells, although this effect may be negligible in comparison with the toxic potential of oxidized derivatives of these compounds [25]. Recent studies on the specific toxicity of naphthalene, biphenyl, anthracene, phenanthrene and others, performed with liposomes as a model system, showed that in the absence of mass transfer limitation, and these compounds do affected the energy transduction throught the membranes [26].

About the medium V, have been supplemented with the soluble substrate sucrose and insoluble babassu oil and pyrene (Table 1). After the inoculations of experiments the growth of *Candida lipolytica* demonstrated a decrease in cells viable numbers until 72 hours like occurred as medium IV (Fig. 4). After this time, the cells numbers increased until at the end of experiments (Fig.5). The pyrene removal rate was 96.87% (Table 2), a inferior value in comparison with medium IV, that presented a mixture of subtrates: soluble, sucrose and insoluble the babassu oil. Similary, both media exhibit a prolongated lag phase, after that the yeast growth increased at the end of assays, in contrast with medium IV, that exhibit only a phase of growth of 24 hours (Fig. 5). In respect to the growth kinetics, medium V, shows a higher specific growth rate (μ) and generation time of 14.43 hours, best condition of growth than medium IV, but the remotion rate of pyrene was inferior .

In some previous studies about this subject, the results are similar to ours. The extent of lag phase exhibit by the yeast possible is due to adaptation period that involve the synthesis ‘de novo’ of enzymes of the cells in presence of pyrene [27]. Recently, some studies have focused about the

ability of yeasts to grow and transformed aromatic compounds is common [28,29], and the cleavage of aromatic structure of the dioxin-like compound dibenzofuran, was characterized by yeast *Trichosporum mucoides* [30]. The use of edible oils such as corn, soy bean, safflower and sunflower oils were used to growth and biosurfactant production by yeast *Torulopsis bombicola* [31] and babassu and corn oil were used as carbon source by *Candida lipolytica* to biosurfactant production [17,32]. Thus, this oils are considered by us a growth substrate. The cells using both substrates, oil and sugar simultaneously, evidently by mutually exclusive mechanisms, thereby achieving increased growth and productivity of antibiotics. The use of oils as carbon sources was for economics reasons, was described in a experiment [33].

However, because the fate of pollutants in the environment is a concern, is important to describe that these are affected by the presence of easily degradable carbon sources and microorganisms utilize a variety of carbon and energy substrates simultaneously: a phenomenon referred to as mixed substrate growth. It is generally considered that a step towards understanding these complex interations: the degradation of mixtures of glucose (an easily degradable substrate) and 3- phenylpropionic acid (3-ppa) (a model pollutant) by *Escherichia coli* ML30. The results indicated that two substrates were always consumed simultaneously [34]. This results are similar to others authors cited in this work. Results previously described, that biosurfactants were produced by *Pseudomonas aeruginosa* 19SJ growing on naphthalene or phenanthrene. This production was observed when cells grown on soluble substrates, such as mannitol or dextrose or on poorly soluble PAHs. The results indicated that an accumulation of naphthalene degradation metabolites was observed when concentrations of biosurfactants in the medium started to increase abruptly. This can be explained by the increasing amount of available naphthalene due to the solubilizing effect of biosurfactants in medium [35]. Similar effects observations in presence

of synthetic surfactants have been reported [36]. We suggest that the high rate of pyrene removal could also be explained by the solubilizing effect of biosurfactants that probably were produced by *Candida lipolytica* in this substrates.

A recent research was to investigate polycyclic aromatic hydrocarbons sorption by bacterial biomass and examine the relationship between PAH biosorption and biodegradation. In this study phenanthrene was used for biosorption studies and pyrene and fluoranthene were used in biodegradation studies. The results suggested that although biosorption can decrease the rate of PAH biodegradation in short term, it can also result in the removal of PAHs. The PAH sorption by the tested bacteria involves a surface adsorption phenomenon rather that a non-specific partitioning into the bacterial cell [37].

However, the use of multiple-substrate utilization by anthracene-degrading *Mycobacterium frederiksbergense* LB501T, were evaluated. The results revealed that this bacteria degrades anthracene and forms biomass from it even in the presence of more readily available dissolved glucose. The capacity of this bacteria to utilize available substrates besides the poorly available PAHs favors the buildup of PAH-degrading biomass. Feeding of supplementary carbon substrates may therefore promote bioremediation, provided that it sustains the pollutant-degrading population rather than other members of the microbial community [38]. The medium I show the higher rate of pyrene removal in cells in decaying phase of growth suggesting that probably occurred the biosortion of this compound by the yeast cells. The yeast biomass may be used as a low-cost, natural and abundant source biosorbent of pollutants compounds. Another works corroborate our results [22,39]. Because the importance of maximing rates of degradation/ remotion in the bioremediation of contaminated sites, further research is required to determine

the relatives roles of biosorption and biodegradation and the microbial physiology of those processes. The strong correlation between carbon sources in the media and the rate of pyrene remotion suggests improved monitoring techniques in future bioremediation applications.

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Table 1. Composition of media used for biodegradation of pyrene by *Candida lipolytica.*

Compounds[a]	g/l	Media				
		I	II	III	IV	V
Yeast extract	5.5	X	X	X	X	X
Urea	3.75	X	X	X	X	X
Potassium phosphate	5.5	X	X	X	X	X
Glucose	10.0	-	X	-	X	-
Sucrose	10.0	-	-	X	-	X
Babassu oil	62.5	X	-	-	X	X
Pyrene	0.01	X	X	X	X	X

[a] X denotes the presence of the compound.

Table 2. Remotion of pyrene by *Candida lipolytica* during the growth in different media.

	Pyrene (%)[a]		Growth kinetics	
Medium	Remaining	Remotion	$\mu\ h^{-1}$ [b]	g (hours)[c]
I [e]	0.98	99.02	[d]	[d]
II [f]	4.22	95.78	0.051	13.58
III [g]	8.22	91.78	0.040	17.32
IV [h]	2.49	97.51	0.032	21.65
V [i]	3.13	96.87	0.048	14.43

[a] Percentage remaining was calculated relative to uninoculated controls after 5 days of incubation.

[b] Specific growth rates (μ) expressed in hours.

[c] generation time (g) expressed in hours.

[d] The exponential 'death' rate was not calculated.

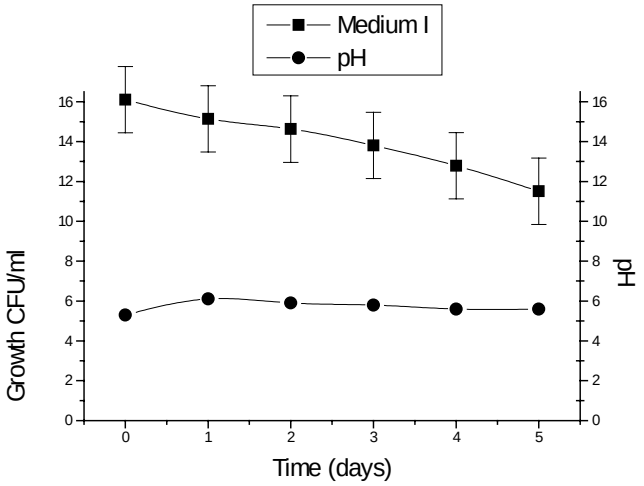
[e] Basal medium supplemented with babassu oil and pyrene.

[f] Basal medium supplemented with glucose and pyrene.

[g] Basal medium supplemented with sucrose and pyrene.

[h] Basal medium supplemented with glucose, babassu oil and pyrene.

[i] Basal medium supplemented with sucrose, babassu oil and pyrene.



. **Figure 1.** Profile of cell growth and pH of *Candida lipolytica* in medium I (yeast extract 5,5 gl^{-1} , urea 3,75 gl^{-1}, potassium phosphate 5,5 gl^{-1}) supplemented with babassu oil 62,5 ml l^{-1} , and pyrene 10mg l^{-1}.
Symbols: ● pH determinations. ■ The growth studies were performed by colony forming units per ml^{-1} . Errors bars indicate the standard deviation on three independent experiments.

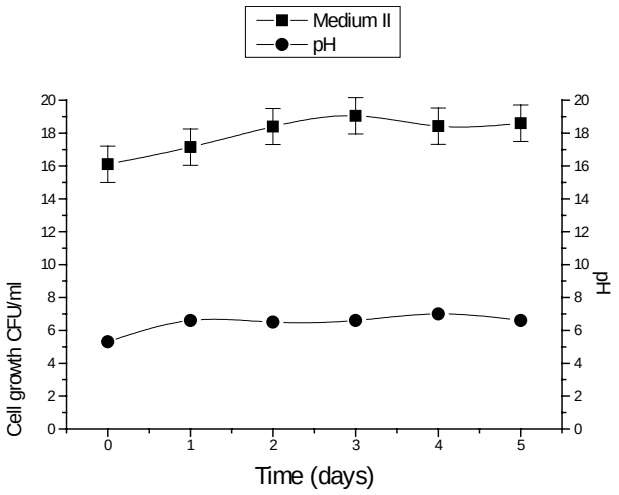


Figure 2. Profile of cell growth and pH of *Candida lipolytica* in medium II (yeast extract 5,5 gl^{-1}, urea 3,75 gl^{-1}, potassium phosphate 5,5 gl^{-1}) supplemented with D-glucose 10 gl^{-1}, and pyrene 10mg l^{-1}.
Symbols: ● pH determinations. ■ The growth studies were performed by colony forming units per ml^{-1}. Errors bars indicate the standard deviation on three independent experiments.

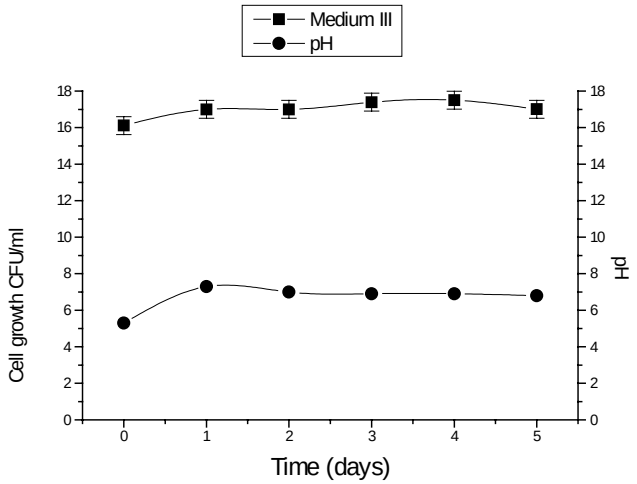


Figure 3. Profile of cell growth and pH of *Candida lipolytica* in medium III (yeast extract 5,5 gl^{-1} , urea 3,75 gl^{-1}, potassium phosphate 5,5 gl^{-1}) supplemented with sucrose 10 gl^{-1} , and pyrene 10mg l^{-1}.
Symbols: ● pH determinations. ■ The growth studies were performed by colony forming units per ml^{-1} . Errors bars indicate the standard deviation on three independent experiments.

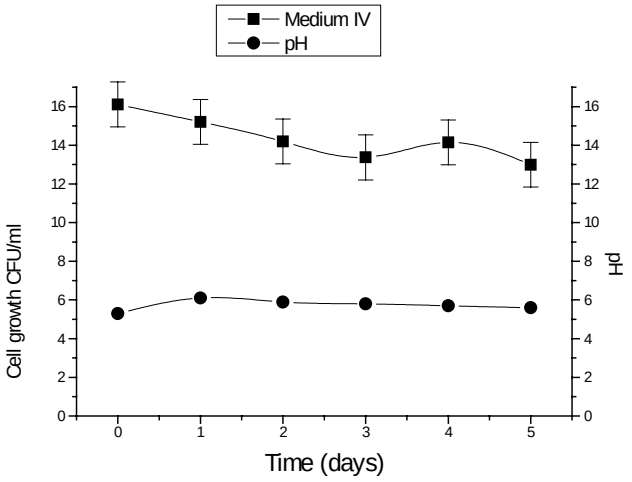


Figure 4. Profile of cell growth and pH of *Candida lipolytica* in medium IV (yeast extract 5,5 gl^{-1} , urea 3,75 gl^{-1}, potassium phosphate 5,5 gl^{-1}) supplemented with D-glucose 10 gl^{-1} , babassu oil 62,5 ml l^{-1} and pyrene 10mg l^{-1}.
Symbols: ● pH determinations. ■ The growth studies were performed by colony forming units per ml^{-1} . Errors bars indicate the standard deviation on three independent experiments.

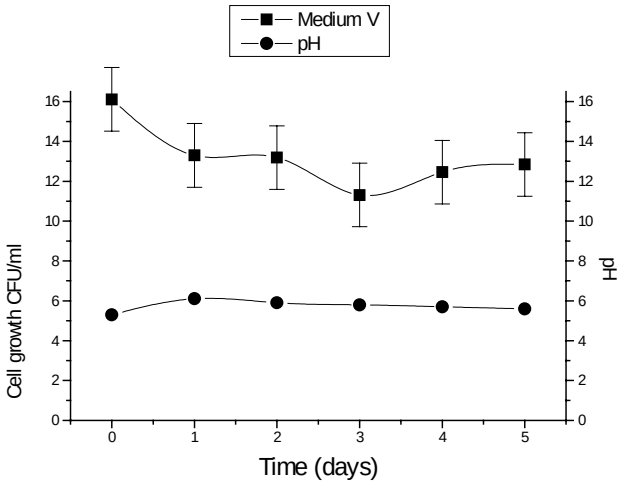


Figure 5. Profile of cell growth and pH of *Candida lipolytica* in medium V (yeast extract 5,5 gl^{-1} , urea 3,75 gl^{-1}, potassium phosphate 5,5 gl^{-1}) supplemented with sucrose 10 gl^{-1} , babassu oil 62,5 ml l^{-1} and pyrene 10mg l^{-1}.
Symbols: ● pH determinations. ■ The growth studies were performed by colony forming units per ml^{-1} . Errors bars indicate the standard deviation on three independent experiments.

CAPÍTULO IV

Biossorção do Pireno por *Candida lipolytica* Imobilizada

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Biossorção do Pireno por *Candida lipolytica* Imobilizada

Vance-Harrop, M.V [1]., Gusmão, N. B[2]., Shiosaki, R. K[3]., Ambrosio, S.T. [4] ,& Campos-Takaki, G. M.[5]

[1] Doutorado em Biologia de Fungos, Universidade Federal de Pernambuco, 50670-420 Recife-Pernambuco, Brasil

[1] LAPA/Recife, Ministério da Agricultura, Pecuária e do Abastecimento.

[2] Departamento de Antibióticos, Universidade Federal de Pernambuco, 50670-420 Recife-Pernambuco, Brasil

[3] Doutorado em Ciências Biológicas, Universidade Federal de Pernambuco, 50670-420 Recife-Pernambuco, Brasil

[4] Centro de Ciências Biológicas, Universidade Federal de Pernambuco, 50670-420 Recife-Pernambuco

[5]Departamento de Química e Núcleo de Pesquisas em Ciências Ambientais,
Universidade Católica de Pernambuco, 50050-590 Recife-Pernambuco, Brasil

Resumo

A biossorção do pireno (derivado do petróleo) foi estudada, utilizando biomassa de *Candida lipolytica* imobilizada em álcool polivinil como matriz inerte. A biomassa imobilizada correspondeu a três concentrações (100, 200, e 300 mg) e o pireno teve como concentração inicial 10mg l^{-1}. A biomassa imobilizada foi empacotada em coluna de vidro e a taxa de eluição foi de 3ml min^{-1}. As frações obtidas foram analisadas por cromatografia em camada delgada e por cromatografia líquida de alta eficiência. A capacidade máxima de biossorção do pireno foi de 86,91, 44,62 e 29,43 mg g^{-1}, respectivamente, para 100mg, 200mg e 300mg de biomassa.

Palavras-chave: Biossorção, Imobilização, *Candida lipolytica,* Pireno.

Introdução.

Biossorção é um termo freqüentemente usado para descrever a sorção e acumulação de produtos químicos por microrganismos. Os mecanismos envolvidos neste processo de acumulação são possivelmente a adsorção na superfície celular ou a absorção por vários componentes intracelulares (8). Nas sociedades modernas, vem aumentando o número de compostos orgânicos danosos eliminados no ambiente, entre estes os hidrocarbonetos aromáticos policíclicos, que são potencialmente mutagênicos e carcinogênicos. A maioria destes compostos está principalmente em resíduos de indústrias, como as de papel, borracha sintética, refinarias de petróleo, tecidos, plásticos e medicamentos (13).

Entre estes compostos estão metais pesados, corantes e derivados aromáticos do petróleo, tratados por métodos de degradação biológica, como adsorção por carbono ativado, extração por solventes, que são tratamentos técnicos de alto custo e impraticáveis, devido à utilização de outros produtos químicos, que também pode causar danos ambientais (2). Microrganismos, como bactérias, fungos e algas podem ser utilizados para remover estes poluentes em soluções aquosas, através da biossorção. No entanto, a biossorção de compostos aromáticos por microrganismos tem sido pouco estudada. Alguns trabalhos realizados sugerem que este processo está associado a alterações nas estruturas da membrana celular pelos compostos e pela interferência nas funções biológicas da membrana (20).

O principal interesse por este processo de biossorção é o baixo custo e a possibilidade de altas taxas de remoção. Devido ao fato de que os poluentes orgânicos hidrofóbicos demonstrarem uma alta tendência a sua acumulação sobre as células microbianas, a biomassa pode ser utilizada como um adsorvente de origem biológica para a remoção de baixas concentrações de compostos orgânicos danosos a saúde (4).

Células vivas e mortas podem ser usadas na remoção de poluentes orgânicos tóxicos. No entanto, a manutenção da biomassa viável durante este processo, apresenta algumas dificuldades, como a necessidade de suplementação com nutrientes e a prevenção de sua toxicidade para os microrganismos. O uso de células mortas na biossorção é mais vantajoso visto que a biomassa não será afetada por resíduos tóxicos e poderá ser regenerada para reutilização (16).

Foi observado anteriormente (17), que a quantidade de poluentes orgânicos acumulados por biomassa microbiana morta foi maior que a acumulação por células vivas. Contudo, o uso de biomassa inativada em forma de pó apresenta alguns problemas como a dificuldade na separação da biomassa após a biossorção e a perda de massa após a adsorção. Portanto, torna-se necessária a utilização de uma matriz polimérica como material de suporte (3). Quando a biomassa é imobilizada, os números de sítios de ligação são facilmente acessíveis aos poluentes em solução, os quais são removidos. O uso de álcool polivinil (PVA) como suporte para imobilização foi iniciado há cerca de 10 anos. O PVA é uma matéria prima de vinylon que é produzido industrialmente com facilidade e em grande quantidade. O PVA oferece também varias vantagens sobre os métodos de imobilização convencionais, como baixo custo, alta durabilidade, estabilidade química e, principalmente, não é tóxico para células viáveis (7,12). Poucos trabalhos a respeito da degradação de compostos tóxicos em resíduos industriais, em células imobilizadas, têm sido encontrados na literatura (11, 19). Entre as leveduras, *Candida utilis* foi estudada quanto a sua habilidade na sorção de vários metais pesados, em células intactas e desidratadas, revelando resultados diferentes para cada tipo de metal e tratamento celular (14).

Este trabalho descreve a remoção do hidrocarboneto aromático policíclico pireno, pela biomassa de *Candida lipolytica* imobilizada em álcool polivinil.

Materiais e métodos

Microrganismo

Candida lipolytica UCP 0065, utilizada neste trabalho, foi mantida em tubos de ensaio contendo Yeast Mold Agar (YMA) , a 4^0C, durante a realização deste trabalho (9).

Meio de cultura e condições de cultivo para o preparo da biomassa

O inóculo para a produção da biomassa foi obtido a partir do YMA. A levedura foi inoculada em Yeast Mold Broth (YMB) e incubada a 28^0C, sob agitação orbital de 150 rpm, durante 24 horas. O meio utilizado para a produção de biomassa foi extrato de malte a 3%. Foram utilizados frascos de Fernbach com capacidade para 2.800 ml contendo 500 ml do meio. O pH foi ajustado em 5.3 e esterilizado. Após a inoculação de 5% de uma suspensão de células com concentração de 10^7 cél./ml os meios foram incubados sob agitação orbital de 150 rpm. Após 48 horas quando as células alcançaram o fim da fase exponencial do crescimento, foram separadas por centrifugação a 1.200x g durante 15 minutos e lavadas 3 vezes em água destilada deionizada. Após este procedimento as células foram liofilizadas.

Imobilização da biomassa

Dois gramas de álcool polivinil foram dissolvidos em 20 ml de água deionizada, a 80^0C logo após 3-5 ml de álcool etílico foram adicionados. A solução do polímero foi resfriada a temperatura ambiente, colocada em agitador magnético durante 16 horas para facilitar a polimerização. Em seguida, a biomassa da levedura foi adicionada em concentrações de 100, 200, e 300mg e homogeneizadas. A solução foi colocada em placas de Petri para secar e em seguida foi triturada em gral, até formar um pó. O pó foi colocado em água durante 3 horas e empacotada em colunas de vidro de 2.2x6 cm. Uma solução de pireno na concentração de $10mgl^{-1}$, foi eluída através da coluna com um fluxo de $3ml\ min^{-1}$. Após a adsorção completa da solução, foram adicionados 35 ml de água deionizada. Uma coluna com PVA foi utilizada como controle.

Extração líquido-líquido e análise em cromatografia em camada delgada (CCD) e cromatografia líquida de alta eficiência (CLAE)

As frações obtidas foram extraídas 3 vezes com acetato de etila. Em seguida, foi adicionada sulfato de sódio anidro para remover qualquer resíduo de água; filtrados e evaporados em roto-evaporador. O extrato obtido foi pré-purificado por CCD em placas de sílica gel (G Merck) utilizando-se como padrão uma solução de pireno. Os compostos foram visualizados através de luz UV (254 nm). Para as análises quantitativas, os compostos identificados nas placas de CCD foram raspados com o auxílio de uma espátula e solubilizados em 20 ml de acetona com pureza para CLAE, centrifugados e concentrados para análise. As amostras foram analisadas para a determinação da concentração residual do pireno, em CLAE, fase reversa, em um sistema Varian de cromatografia líquida consistindo de: coluna C_{18}, detector de UV-VIS modelo 320, sistema de liberação de solvente modelo 210. Este sistema foi controlado pelo software Varian Star (versão 5.31). A fase móvel utilizada foi a acetonitrila e os resultados foram expressos como o percentual de pireno residual.

Determinação da dessorção do pireno

A dessorção do pireno foi conduzida pela extração a partir da biomassa imobilizada utilizando acetato de etila por 3 vêzes. Em seguida os extratos obtidos foram analisados por CCD e CLAE para determinação da concentração do pireno residual e dessorvido.

Determinação da capacidade de biossorção do pireno (mg g^{-1})

A capacidade de biossorção do pireno pela biomassa de *Candida lipolytica* imobilizada em álcool polivinil, foi determinada pela medição da concentração do composto na fase aquosa antes e após o contato com o biosorvente e foi expressa de acordo com Jeon et al. (2002), através da seguinte fórmula:

$Q = C_i V_i - C_f V_f / m,$

onde Q é capacidade de sorção do pireno (mg g^{-1}), C_i é a concentração inicial do pireno (mg l^{-1}), V_i é o volume inicial (l^{-1}), C_f é a concentração final do pireno (mg l^{-1}), V_f é o volume final (l^{-1}), e m = a biomassa imobilizada inicial (g). Após a completa eluição da solução do pireno, o efluente da coluna foi coletado para as análises.

Resultados e Discussão

Em estudos previamente realizados, *Candida lipolytica* demonstrou alta capacidade de biossorção sob diferentes condições de cultivo (1). A presença do composto no eluente foi detectada em pequenas quantidades. A eficiência da coluna demonstrada nas figuras 1 e 2, onde a capacidade máxima de biossorção do pireno foi 86,91 mg g^{-1}, com a coluna preparada com 200mg de biomassa imobilizada mostrou a biossorção de 44,62 mg g^{-1} e a coluna contendo 300 mg apresentou uma capacidade máxima de 29,43 mg g^{-1}. Os valores em percentagem do pireno residual foram os seguintes, na coluna contendo 100 mg de biomassa imobilizada, apresentou 13,19% de pireno residual, a coluna com 200 mg, 10,85% e a coluna contendo 300 mg mostrou o percentual de 11,81. A dessorção do pireno apresentou um percentual de cerca de 2% indicando que ocorreu uma forte sorção pela biomassa. Trabalhando com biossorção de metais, alguns autores descreveram resultados similares aos deste estudo, encontrando a capacidade máxima de biossorção, na menor concentração de biomassa (18). A concentração do biosorvente tem sido descrito como um fator importante no processo de biossorção. Os resultados obtidos sugerem que a capacidade máxima de biossorção diminuiu quando a concentração de biomassa foi aumentada e este fenômeno parece ocorrer aos valores mais baixos de concentração de biomassa. Alguns autores, esclareceram este processo, hipotetizando que um aumento na concentração da biomassa, leva a uma interferência entre os sítios de ligação (10).

Estudos sobre a biossorção de hidrocarbonetos aromáticos policíclicos em biomassa imobilizada não foram encontrados na literatura. No entanto, alguns autores relatam estudos utilizando a sorção com sorventes não biológicos, como bentonita ativada, para poluentes orgânicos nocivos, como compostos fenólicos (5).

Experimentos de biossorção foram conduzidos biomassa coletada em poço arenoso poluído por consecutivos derramamentos acidentais de fenol, 2-monoclorofenol, 2,4,6-triclorofenol e pentaclorofenol sob condições de biorremediação. Os resultados demonstraram que a biossorção destes compostos pela biomassa selvagem foi processada rapidamente em um período de aproximadamente duas horas (6). Contudo, estudos relataram que a biomassa morta de *Rhizopus arrhizus* alcançou uma alta biossorção de pentaclorofenol em relação a biomassa viva deste fungo. Células imobilizadas de *Trichosporon cutaneum* R57 em um sistema de resina (Amberlite HAD-4) mostraram grande habilidade em degradar compostos tóxicos em comparação com as células livres (2). Experimentos realizados com biomassa de *Bacillus insolitus* em alginato de sódio, demonstraram que em baixas concentrações de 2,4-diclorofenol (10-50 mg l^{-1}), a biomassa imobilizada mostrou uma alta remoção do composto do que as células de *B. insolitus* em suspensão (19). A investigação da biossorção do fenol por biomassa de *Aspergillus niger* em solução aquosa, foi realizada em uma coluna de polisulfona com uma concentração inicial de 1000 μg l^{-1} de fenol. Os resultados descritos, indicaram que aproximadamente 66% do fenol foi removido pela coluna, e a dessorção deste composto foi de aproximadamente 5% (15).

Os resultados destes experimentos demonstram que a biomassa de *Candida lipolytica* apresenta grande potencial de utilização como um biosorvente para a remoção de hidrocarbonetos aromáticos policíclicos, como o pireno. A aplicação de biomassa da levedura apresenta grande potencial por ser natural e abundante podendo ser utilizada para a remoção de compostos orgânicos tóxicos, em baixas concentrações ou após o tratamento físico-químico convencional.

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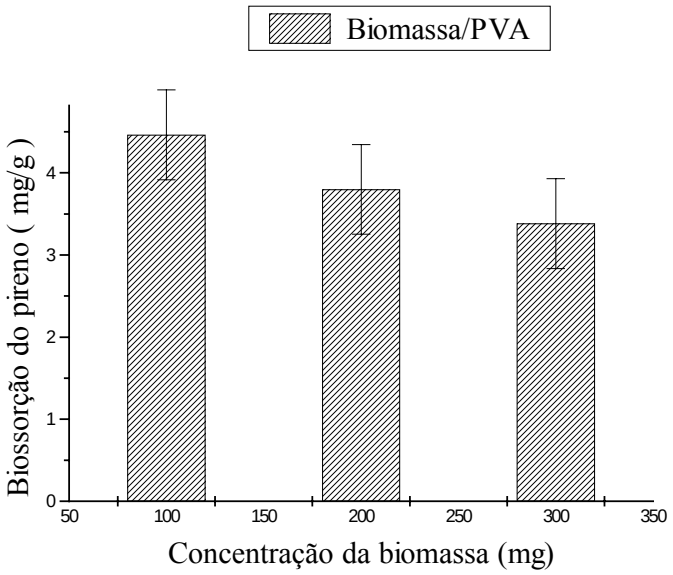

Figura 1. Capacidade máxima de biossorção do pireno por *Candida lipolytica* imobilizada em mg g^{-1}.
Os dados são valores da média de determinações em duplicata.

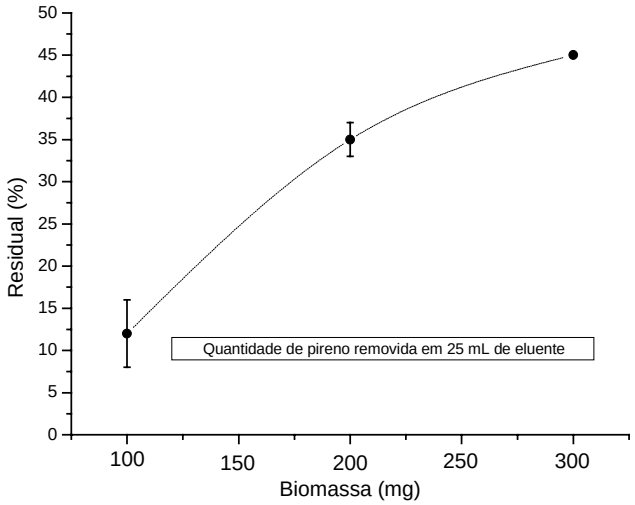

Figura 2. Concentração residual do pireno em percentual, em 25 ml de eluente; de colunas com 100, 200, e 300 mg de *Candida lipolytica* imobilizada.

CONCLUSÕES GERAIS

CAPÍTULO I

- *Candida lipolytica* produz novos bioemulsificantes extracelulares com atividade de emulsificação em fermentação por batelada utilizando óleo de babaçu e D-glicose como fontes de carbono.
- O meio basal, constituído por uréia, sulfato de amônio e fosfato de potássio, tendo como base mineral a água do mar natural diluída a 50%, demonstrou excelente condições de produção de biopolímeros e baixo custo.
- A produção dos novos biopolímeros não é afetada em meio de cultura com salinidade de 13% e suas moléculas são constituídas por carboidratos, proteínas e lipídeos.

CAPÍTULO II

- A composição de meios de cultura para a produção de biossurfactantes por *Candida lipolytica*, através de planejamento fatorial em três etapas, mostrou um aumento na produção de surfactantes com atividade de emulsificação em relação aos meios utilizados no capítulo I.
- Extrato de levedura, uréia, e fosfato de potássio em suas concentrações mais altas são mais eficientes na produção de biossurfactantes.
- O sulfato de amônio é indicado em seu nível mais baixo e o óleo de babaçu é independente quanto ao seu nível, no aumento da produção de surfactantes com atividade de emulsificação.

CAPÍTULO III

- A remoção e biossorção do pireno ocorrem em condições de interações de substratos mistos, solúveis (glicose e sacarose) e insolúveis (óleo de babaçu e pireno).
- A taxa de remoção do pireno mais alta (99,02%) ocorre pelo processo de biossorção por células em fase de declínio, no meio contendo apenas substratos insolúveis como fonte de carbono.
- A condição de crescimento em substratos mistos solúvel (glicose) e insolúvel (óleo de babaçu) foi mais eficaz quanto ao crescimento celular, alcançando também uma alta taxa de remoção (97,51%) de pireno.

CAPÍTULO IV

- *Candida lipolytica* imobilizada em álcool polivinil como matriz inerte apresenta grande capacidade de biossorção do hidrocarboneto aromático policíclico pireno.
- O pireno foi removido pela biomassa, cuja capacidade máxima de biossorção deste composto foi de 86,91 mg g^{-1} na coluna com 100 mg de biomassa sendo este o melhor resultado.
- A dessorção do pireno foi de aproximadamente 2% , indicando uma forte biossorção pela biomassa.

ANEXOS

Normas utilizadas

CAPÍTULO II

Bioresource Technology

Guide for Authors

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References should be given in the following form:
Deka, G.C., 1987. Physical, chemical and pulping characteristics of hybrid *Salix* clones. PhD thesis, Faculty of Forestry, University of Toronto.
Gandini, A., 1992. Polymers from renewables resources. In: Aggraval, S. L., Russo, S. (Eds.), Comprehensive Polymer Science. Pergamon Press, Oxford, (Chapter 10).
Hobson, P.N., 1969. Growth of mixed cultures and their biological control. In: Meadow, P.M., Pirt S.J. (Eds.), Microbical Growth. Proc 9th Symp Soc Gen Microbiology. Cambridge University Press, London, pp. 43-64
Vassileva, M., Azcon, R., Barea, J.-M., Vassilev, N., 1999. Effect of encapsulated cells pf Enterobacter sp on plant growth and phosphate uptake. Bioresource Technol 67 (3), 229-232.
Zakis, G., Neiberte, B., 1993. Amines derivatives of lignin - a method for obtaining an acid sorbent. LV Patent 5213.
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CAPÍTULO III

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GuideforAuthors

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Enzymes. For enzymes, use the recommended (trivial) name assigned by the Nomenclature Committee of the International Union of Biochemistry 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 for Nomenclature Committee of the International Union of Biochemistry. (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 at least ten times in the text (including Tables and Figure legends).
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.
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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.

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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 two copies are enclosed along with the submitted manuscript. References to books should include the title of the book, the year of publication, the publishing company and the place of publication.
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[1] Stambuk, B.U. and Araujo, P.S. (2001) Kinetics of active a-glucoside transport in Saccharomyces cerevisiae. FEMS Yeast Res. 1, 73-78.
[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.
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CAPÍTULO IV

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b. Paper or chapter in a book
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c. Book
Salyers, A.A.; Whitt, D.D. *Bacterial pathogenesis. A molecular approach.* ASM, Washington, 1994, 418p.

d. Patent
Hussong, R.V.; Marth, E.H.; Vakaleris, D.G. Manufacture of cottage cheese. *U.S. Pat. 3,117,870*. Jan.14, 1964.

e. Thesis
Calzada, C.T. Campylobacter jejuni *e* Campylobacter coli – *caracterização em sorogrupos e biotipos das cepas isoladas no município de São Paulo no período de 1983-1989*. São Paulo, 1991, 131p. (Ph.D. Thesis. Instituto de Ciências Biomédicas. USP).

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Simões, G.S.; Silva, J.; Toledo, A.S.; Gontijo Filho, P.P. *Micobactérias não tuberculosas isoladas de pacientes com síndrome da imunodeficiência adquirida*. XVII Congresso Brasileiro de Microbiologia, Santos, 1993, p.41.

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