UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
MESTRADO EM BIOQUÍMICA

BIOPRODUÇÃO DE METABÓLITOS DE *Canoparmelia texana* (TUCK.) ELIX & HALE A PARTIR DE IMOBILIZAÇÃO CELULAR

MESTRANDA: ALBA TATIANA SERAFIM DO NASCIMENTO

ORIENTADOR: PROF. DR. NICÁCIO HENRIQUE DA SILVA

CO-ORIENTADOR: PROF. DR^{a}. EUGÊNIA CRISTINA GONÇALVES PEREIRA

RECIFE, 2007

ALBA TATIANA SERAFIM DO NASCIMENTO

BIOPRODUÇÃO DE METABÓLITOS DE *Canoparmelia texana* (TUCK.) ELIX & HALE A PARTIR DE IMOBILIZAÇÃO CELULAR

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

NASCIMENTO, Alba Tatiana Serafim do

Bioprodução de metabólitos de *Canoparmelia texana* (Tuck.) Elix & Half a partir de imobilização celular / Alba Tatiana Serafim do Nascimento. – Recife: A Autora, 2007.

ix,58 p.: il.

Dissertação (Mestrado em Bioquímica) – UFPE. CCB

1. Líquen 2. *Canoparmelia texana* 3. Ácido divaricático 4. Imobilização celular. I. Título

582.29	CDU (2ª. Ed.)	UFPE
579.7	CDD (22ª. Ed.)	CCB – 2007 – 134

Ata da defesa de dissertação da Mestranda **Alba Tatiana Serafim do Nascimento,** realizada em 17 de janeiro de 2007, como requisito final para obtenção do título de Mestre em Bioquímica.

Às 09:10 (nove horas e dez minutos), do dia 17 de janeiro de 2007, foi aberto, no Auditório Prof. Marcionilo de Barros Lins – Depto. de Bioquímica/CCB/UFPE, o ato de defesa de dissertação da mestranda **Alba Tatiana Serafim do Nascimento**, aluna do Curso de Mestrado em Bioquímica/CCB/UFPE. Iniciando os trabalhos o Prof. Dr. Nicácio Henrique da Silva, fez a apresentação da aluna, de seu orientador ele próprio, da co-orientadora Profa. Dra. Eugênia Cristina Pereira e da Banca Examinadora composta pelos professores doutores: Nicácio Henrique da Silva, na qualidade de Presidente, Luana Cassandra Breitenbach Barroso Coelho, Maria da Paz Carvalho da Silva, ambos do Depto. de Bioquímica/CCB/UFPE, e Emerson Peter da Silva Falcão, do Centro Acadêmico de Vitória/UFPE. Após as apresentações, o Prof. Dr. Nicácio Henrique da Silva convidou a aluna para a apresentação de sua dissertação intitulada: **"Bioprodução de Metabólitos de *Canoparmelia texana* (TUCK.) Elix & Hale a partir de Imobilização Celular** "e informou que de acordo com o Regimento Interno do Curso, a candidata dispõe de até 50 (trinta) minutos para apresentação do trabalho e o tempo de argüição para cada examinador, juntamente com o tempo gasto pelo aluno para responder às perguntas será de 30 (trinta) minutos. A aluna procedeu a explanação e comentários acerca do tema em 35 (trinta e cinco) minutos. Após a apresentação da mestranda, o Sr. Presidente convidou a Banca Examinadora para ocupar seus lugares e passou a palavra ao primeiro examinador, o Prof. Dr. Emerson Peter da Silva Falcão, que agradeceu o convite, fez alguns comentários e sugestões, e iniciou sua argüição. Ao final, o referido professor deu-se por satisfeito. Em seguida, o Sr. Presidente passou a palavra para Profa. Dra. Luana Cassandra Breitenbach Baroso Coelho que agradeceu o convite, fez alguns comentários e deu algumas sugestões, iniciando sua argüição. Ao final, a referida professora deu-se por satisfeita. Daí o Sr. Presidente passou a palavra para a Profa. Dra. Maria da Paz Carvalho da Silva, que agradeceu o convite, fez alguns comentários e deu algumas sugestões, iniciando sua argüição. Ao final, a referida professora deu-se por satisfeita. Em seguida, o Sr. Presidente usou da palavra para tecer alguns comentários, agradecer à Banca Examinadora e parabenizar a candidata. Daí o Sr. Presidente passou a palavra para a co-orientadora que fez alguns esclarecimentos. Finalmente, a sessão foi suspensa para proceder ao julgamento pela Banca Examinadora, a qual se reuniu na Secretaria do Curso. Após alguns comentários, a Banca decidiu, por unanimidade, conceder a menção "**Aprovada com Distinção**". Nada mais havendo a tratar, lavrei a presente ata que vai assinada por mim, Secretário, e demais membros da Banca Examinadora. Recife, 17 de janeiro de 2007.

Emerson Peter da Silva Falcão.

SUMÁRIO

AGRADECIMENTOS

A Deus, por tudo que me ofertou durante toda minha vida.

Aos meus pais – Luiz Carlos Serafim e Maria Helena Serafim – pelo amor, dedicação, empenho e inexplicável esforço concedidos para a realização de meus objetivos e sonhos.

Ao meu amor – Gustavo Dimech – por todo apoio, companheirismo, paciência e incentivos concedidos durante a realização de mais esse sonho.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pela concessão de bolsa de estudo para a realização do presente trabalho.

Ao Departamento de Bioquímica da Universidade Federal de Pernambuco, pelo apoio para a realização do curso de mestrado.

Aos meus orientadores minha eterna gratidão. Dr. Nicácio Henrique da Silva, pela orientação científica, dedicação, apoio, compreensão, paciência, e amizade e Dra. Eugênia Cristina Gonçalves Pereira pela competência, estímulo, otimismo, confiança e inestimável orientação proporcionada desde o início de minha vida científica.

A todos que fazem parte do Laboratório de Produtos Naturais, pelo companheirismo e auxílios prestados.

A João Virgínio, pelo apoio dispensado em todos os momentos solicitados.

Aos meus colegas do Curso de Mestrado em Bioquímica, em especial, à Paula, Cynthia, Nyce e Flávia pela amizade e ajuda em todos os momentos.

LISTA DE FIGURAS

3. CARACTERIZAÇÃO MORFOLÓGICA E QUÍMICA DA ESPÉCIE ESTUDADA

7. TRABALHOS A SEREM SUBMETIDOS À PUBLICAÇÃO

7.1 Capítulo I

7.2 Capítulo II

RESUMO

Este estudo teve como objetivo produzir metabólitos de *Canoparmelia texana* (Tuck.) Elix & Hale a partir da imobilização de células e fragmentos do talo *in natura,* através do uso de biorreatores com sistema fixo (tradicional), em movimento, e sob fluxo contínuo. Células extraídas de *C. texana,* ou fragmentos do talo foram imobilizados, separadamente, em biorreatores utilizando a caulinita como matriz de enclausuramento e acetato de sódio a 0,1, 1,0 e 10,0 mM, como precursor biossintético das substâncias típicas da espécie. Alíquotas retiradas, a diferentes intervalos de tempo, foram extraídas com éter/acetato de etila (65:35,v/v) e clorofórmio/acetonitrila (60:40, v/v), e mensuradas em espectrofotômetro a 254 e 366 nm. Os extratos, após evaporados, foram avaliados por cromatografia em camada delgada (CCD). As leituras espectrofotométricas dos extratos orgânicos revelaram a síntese de substâncias, pelas células e fragmentos do talo imobilizados, em todos os sistemas de imobilização e, em todas as concentrações do precursor. Houve predominância quantitativa das substâncias bioproduzidas pela imobilização dos fragmentos do talo no sistema em movimento. Estes e as células de *C. texana,* imobilizados nos diferentes sistemas, bioproduziram as mesmas substâncias contidas no talo *in natura*, indicando, além da adaptação da espécie em estudo ao processo de imobilização celular, uma promissora estratégia biotecnológica de produção de atranorina e ácido divaricático, duas substâncias com inúmeras aplicações biológicas.

Palavras-chave: líquen, ácido divaricático, atranorina, *Canoparmelia texana,* imobilização celular

ABSTRACT

This study aimed the production of *Canoparmelia texana* (Tuck.) Elix & Hale metabolites from the immobilization of cells and *in natura* thallus fragments, through the use of bioreactors with fixed (traditional) system, moving system, and continuous-flow system. *C. texana* extracted cells, or thallus fragments, were immobilized separately, in bioreactors using kaolinite as the entrapment matrix and sodium acetate at 0.1, 1.0 and 10.0 mM, as the biosynthetic precursor of typical substances of the species. Samples taken at different periods of time were extracted with diethyl ether/ethyl acetate (65:35, v/v) and chloroform/acetonitrile (60:40, v/v), and measured in a spectrophotometer at 254 and 366 nm. After evaporation, extracts were assessed through thin layer chromatography. The organic extract spectrophotometric measuring revealed the synthesis of substances by cells and thallus immobilized fragments in all immobilization systems, and in all concentrations of the precursor. There was a quantitative predominance of bioproduced substances by thallus immobilized fragments in the moving system. These and the *C. texana* cells, immobilized in the different systems, bioproduced the same substances found in the *in natura* thallus, indicating not only the adaptation of studied species to the process of cellular immobilization, but a promising source of biotechnology for the production of atranorin and divaricatic acid, two substances with countless biological applications.

Key words: lichen, divaricatic acid, atranorin, *Canoparmelia texana*, cellular immobilization

1 INTRODUÇÃO

O interesse por substâncias liquênicas tem aumentado sensivelmente pela comunidade científica que atua na farmacologia de produtos naturais, uma vez que são evidentes seus efeitos terapêuticos (HALE-JR.,1983; PEREIRA, 1998).

De acordo com a literatura, liquens são utilizados desde a antiguidade como plantas medicinais (ABRAHAN & FLOREY, 1949), produzem óleos essenciais e substâncias fixadoras de perfume, corantes de tecidos, graxas e óleos. São utilizados na manufatura de cerveja, conhaque e álcool (LLANO, 1951); seus metabólitos são ativos contra fungos e bactérias (PEREIRA *et al.*, 1996), tumores e células cancerígenas (LIMA *et al.*, 1990; PEREIRA *et al.*, 1994a), além de possuírem ação alelopática (LAWREY, 1977; YANO, 1994), serem eficientes no controle biológico de insetos (COSTA FILHO *et al.*, 1991) e, possuírem importante papel ecológico, uma vez que podem ser utilizados como bioindicadores e biomonitores de poluição ambiental (ADAMO *et al.*, 2003; GUIDOTTI *et al.*, 2003; WALKER *et al.,* 2003; MAZZITELLI *et al.,* 2006).

Diante de uma diversidade de utilizações dos metabólitos liquênicos na indústria farmacêutica, de cosméticos, têxtil e de alimentos, sua aplicação ao nível comercial deve ser criteriosa. Isto se deve ao fato da necessidade de destruição de uma grande quantidade de biomassa, dificilmente renovável, para a obtenção de um composto de interesse econômico (VICENTE *et al.*, 1995).

As técnicas para isolamento de compostos fenólicos proporcionam baixo rendimento, pois dependem do procedimento utilizado e dos solventes empregados. Algumas espécies também dependem do período coletado, visto que só produzem determinado fenol em certo período do ano (HALE-JR., 1983; PEREIRA, 1989).

Através de imobilizações celulares e enzimáticas, é possível a obtenção contínua de compostos liquênicos a partir do talo *in natura,* impedindo a destruição de grande quantidade de biomassa. A continuidade destes estudos visa a produção destes metabólitos, em quantidades que justifiquem o processo, permitindo seu uso a nível de investigação e/ou industrial.

2 REVISÃO DA LITERATURA

2.1 Liquens e seus compostos

Os liquens são pouco estudados e, na maioria dos casos, são conhecidos por sua capacidade de habitar substratos rochosos, quando iniciam o processo de pedogênese. No entanto, este grupo biológico tem inúmeras utilidades econômicas, algumas delas medicinais, além de uma interação notável com os elementos do ambiente.

Esses seres são resultantes de uma associação simbiótica entre alga e fungo, cujas naturezas distintas resultam em um talo de estrutura estável, onde o fungo é o exo-habitante. A simbiose entre componentes tão distintos, como a alga, que é clorofilada, e, portanto, fotossintética, e o fungo, aclorofilado e heterótrofo, confere ao líquen um funcionamento ímpar, não comparado a outros grupos taxonômicos (NASH III, 1996).

A fotossíntese da alga liquênica, ou fotobionte, proporciona os hidratos de carbono necessários para o início da nutrição, e todas as reações metabólicas do líquen. Estes açúcares são repassados ao fungo, que não têm capacidade de sintetizá-los, pois não são seres fotossintéticos. A partir desse transporte massivo de carboidratos, são sintetizadas as substâncias liquênicas, produtos finais do processo, únicas desses seres, e responsáveis pelos benefícios advindos dos liquens (CULBERSON *et al*., 1977; MACFARLANE & KERSHAW, 1984). Estas substâncias são extracelulares, organizam-se sob a forma de cristais insolúveis em água, geralmente depositados sobre a hifa do micobionte, sendo designados como ácidos liquênicos. São característicos do grupo e, são, na maioria, fenóis, para e meta depsídeos, depsidonas, terpenóides, esteróides entre outros, tais como os ácidos úsnico, barbático, estístico, nortístico e fumarprotocetrárico (HALE-JR, 1983; NASH III, 1996; HONDA & VILEGAS, 1999).

A maioria desses metabólitos são compostos provenientes de rotas metabólicas específicas, tais como: 1- via do acetato polimalonato, onde são sintetizadas substâncias típicas, como quinonas, depsídeos, depsidonas, ácidos graxos e dibenzofuranos; 2- via do ácido mevalônico, onde ocorre a formação dos terpenóides e esteróis; 3- via do ácido chiquímico que origina os pigmentos amarelos e 4- via dos aminoácidos (VICENTE, 1975; HONDA, 2006).

2.2 Imobilização celular

Apesar de mostrarem-se promissoras, as técnicas de cultivo *in vitro*, bem como as de síntese do talo liquênico, não apresentaram até o momento resultados que as justifiquem.

Ambas metodologias não chegaram à reprodução do líquen em sua forma natural. O micobionte *Lecanora chrysoleuca* unido, em laboratório, ao ficobionte *Pseudotrebouxia*, desenvolveu um talo que produzia ácido úsnico, mas não o ácido pseudoplacodiólico, apesar de ambos fenóis serem produzidos pelo talo *in natura* (CULBERSON & AHMADJIAN, 1980). Por outro lado, as técnicas de cultura de tecidos mais elaboradas, as quais objetivam produção de fenóis liquênicos, não apresentam relação entre os fenóis produzidos pela cultura e aqueles que ocorrem no talo em estado natural (YOSHIMURA *et al.*, 1993).

Uma alternativa para produção de substâncias liquênicas para fins industriais e científicos, sem a destruição da micota liquenizada, é o uso da imobilização celular. Neste recurso biotecnológico, as células encontram-se aprisionadas em espaço definido, tendo sua atividade total ou parcialmente mantida para uso continuado e repetido. É uma atraente alternativa ao uso de sistemas enzimáticos purificados, uma vez que a própria célula organiza e fornece as condições ideais de funcionamento, além das vantagens de reutilização e economia de tempo (YAMAMOTO *et al.*, 1976; SVITEL *et al.*, 1998).

Diferentes métodos, utilizando diversas matrizes, são conhecidos para imobilizar enzimas, tais como: enclausuramento, adsorção ou ligação covalente (SKRYABIN & KOSHCHEEHKO, 1987). No que diz respeito à matriz, características como afinidade para ligação, ausência de efeitos tóxicos, resistência mecânica e à degradação, devem ser consideradas durante sua seleção (GBEWONYO *et al.*, 1987).

O primeiro trabalho de imobilização de uma enzima liquênica se deve a Mosbach & Mosbach (1966). Uma orselinato descarboxilase de *Umbilicaria pustulata* foi imobilizada em poliacrilamida a 5 e a 20%, posteriormente granulada, e posta em contato com ácido orselínico para estimular desprendimento de CO_2. A atividade da enzima imobilizada a 20% permanecia praticamente constante em 14 dias de armazenamento, o que não ocorria com a enzima imobilizada a 5%.

Por este procedimento foram imobilizadas células intactas do mesmo líquen, através do qual foi demonstrado que a atividade descarboxilante era mantida por dois meses de armazenamento a 20 ^{o}C. A imobilização dessas células oferecia a possibilidade de estudar reações em cadeia. Assim, a atividade descarboxilante poderia ser detectada acrescentando ao reator os ácidos orselínico ou lecanórico como substrato. Neste caso, a orselinato depsídeo hidrolase contida nas células produzia duas moléculas de ácido orselínico, por cada molécula de ácido lecanórico hidrolisada (MOSBACH, 1983).

A mesma enzima, por ser uma das mais estudadas do metabolismo dos fenóis, foi também utilizada em estudos de imobilização por Garcia-Junceda & Vicente (1986), que descreveram um método de imobilização desvitalizante usando talos de *Pseudevernia furfuracea,* tendo poliacrilamida como matriz. Foi observada uma alta atividade enzimática nesta imobilização, já que 90% do ácido evérnico adicionado ao biorreator era eficientemente hidrolisado. Não foi detectada atividade orselinato descarboxilase, já que não aparecia orcinol nos eluatos. No entanto, a poliacrilamida plasmolizava as células, reduzindo sua vitalidade quando imobilizadas. Por isso, células de *Evernia prunastri* foram imobilizadas em alginato. Este processo apresentou a vantagem de não ser desvitalizante, permitindo que as células mantivessem suas funções vitais por tempo indeterminado (GONZALEZ *et al.*, 1984). Outra imobilização em alginato foi realizada por Vicente & Molina (1993), utilizando células de *Xanthoria parietina* para obtenção de produtos de degradação da parietina. Por meio de microscopia eletrônica de transmissão foi constatado que neste tipo de matriz, as células foram capazes de se dividirem ativamente.

O conhecimento de que, ao receberem a adição de substrato, enzimas presentes em células imobilizadas são capazes de iniciar processo de degradação e elaboração de produtos de reação enzimática, favoreceu a hipótese de que estas células, quando imobilizadas e suplementadas com precursor enzimático e, não com substrato, produziriam substâncias típicas de suas rotas metabólicas.

Neste sentido, com a finalidade de produzir açúcares alcoólicos de liquens, Pereira *et al.* (1995a) imobilizaram células de *Cladonia verticillaris* em alginato de cálcio, utilizando bicarbonato de sódio como precursor. As células aprisionadas foram mantidas sob constante iluminação por mais de 15 dias. Os polióis e açúcares liberados para o meio foram extraídos e analisados por cromatografia líquida de alta eficiência (CLAE). Foi comprovado que houve maior produção de ribitol e glicose quando as células eram incubadas em bicarbonato a 10,0 mM. Entretanto, o sal sódico utilizado provocou o enfraquecimento da estrutura das esferas de alginato endurecidas pelo cálcio, diluindo-as em menos de cinco dias. Por outro lado, as mantidas em solução do mesmo precursor a 1,0 mM foram conservadas por período mais longo, com produção e excreção apenas do ribitol. Ainda que as células imobilizadas conservassem sua vitalidade, os fotobiontes apresentaram seus cloroplastos desorganizados. A falta de produção de manitol por parte do fungo, a partir do ribitol translocado da alga, indicou que o contato entre os simbiontes foi prejudicado durante a imobilização.

Diversos liquens presentes no nordeste brasileiro têm sido estudados no intuito de avaliar as espécies eficientes para esta metodologia de bioprodução de metabólitos. Para contornar o problema da dissolução das esferas de alginato endurecidas, o que impossibilitava a determinação da concentração ideal de precursor para a bioprodução optou-se pelo uso da caulinita. Trata-se de um silicato do grupo do caulim, produto da decomposição de feldspatos. É uma argila de estrutura cristalina constituída de partículas finíssimas de natureza coloidal, de composição química $Si_4O_{10}(OH)_8Al_{14}$ (LEINZ & AMARAL, 1978). Este tipo alternativo de matriz de imobilização reduziu o custo final dos experimentos, sem perda da eficiência e da produtividade celular, com estabilidade mecânica diante das soluções de concentrações elevadas, e sem os inconvenientes da desvitalização celular. Assim, com o intuito de se testar nova matriz de imobilização, células de *Cladonia substellata* foram imobilizadas em caulinita, e produziram o ácido úsnico, composto liquênico de grande importância medicinal (PEREIRA *et al.*, 1995b). Outras espécies da família Cladoniaceae vêm sendo estudadas com este mesmo objetivo. *C. corallifera* procedente da Amazônia produziu os ácidos úsnico e didímico (PEREIRA *et al.*, 1999a), enquanto que *C. verticillaris* de tabuleiros arenosos da Paraíba produziu a atranorina, e não o ácido fumarprotocetrárico, seu componente principal (PEREIRA, 1998). O mesmo aconteceu com *C. clathrata*, que tem ácido fumarprotocetrárico como fenol majoritário. Neste caso, foi produzido o ácido hipoprotocetrárico e seu aldeído, notoriamente na concentração de 1,0 mM do precursor utilizado, o acetato de sódio (PEREIRA *et al.*, 1999b).

Liquens da Caatinga tiveram suas células imobilizadas. *Ramalina aspera*, ocorrente no município de Venturosa (PE), revelou uma produção contínua de metabólitos e a concentração do precursor mais satisfatória foi de 1,0 mM. Esta espécie produziu efetivamente o ácido úsnico, um potente bactericida, substância liquênica mais estudada no ponto de vista químico e de bioatividade (SANTOS *et al.*, 2000).

Pseudocyphellaria aurata, espécie típica de Brejos, produziu eficientemente metabólitos, a partir de imobilização de suas células, com biossíntese das mesmas substâncias produzidas pelo talo *in natura*. Foram detectados o ácido pulvínico e seus derivados, como a dilactona pulvínica e a calicina, não havendo interferência das concentrações utilizadas do precursor, acetato de sódio, nos ensaios de bioprodução (ALBUQUERQUE *et al.*, 2000).

Células imobilizadas de *Heterodermia leucomela*, coletada em área de Caatinga, também produziram os compostos majoritários desta espécie, que são a atranorina, o ácido salazínico e a zeorina, além de outras substâncias não identificadas (ROCHA, 1999).

O estado de fertilidade também é um dos fatores que influencia a produtividade de compostos fenólicos através de células imobilizadas. Isto foi constatado quando Pereira *et al.* (2002) imobilizaram células de *Cladia aggregata* nos estados fértil e estéril. Esta espécie apresentou maior produtividade quando estéril, em relação às amostras férteis. Em ambos os casos foi produzido o composto majoritário da espécie, o ácido barbático.

Nóbrega (2002), através de sistema fixo, imobilizou células de *Parmotrema andinum* em caulinita, produzindo metabólitos distintos dos sintetizados pelo talo *in natura*. A concentração do precursor não influenciou na bioprodutividade e, as células mantiveram sua vitalidade por 31 dias.

Até o momento foi constatado que, de forma geral, algumas espécies têm produção de compostos semelhantes aos obtidos do talo *in natura*, por suas células imobilizadas; outras sintetizam produtos intermediários de suas rotas metabólicas, ou seus fenóis na forma reduzida (PEREIRA *et al.*, 1999b). Fontaniella *et al.* (2000) comprovaram que tal fato ocorre em algumas espécies pela perda total de contato entre os simbiontes, o que dificulta a troca de co-fatores entre eles.

Atualmente objetiva-se, além da síntese de compostos naturais dos liquens, uma maior eficiência dos sistemas imobilizados. Lima (2004) comparou os resultados obtidos por Pereira *et al.* (1995b), desenvolvendo experimento em distintos biorreatores. Foi possível comprovar a influência da concentração do precursor e o seu fluxo contínuo na eficiência de produção do ácido úsnico de *Cladonia substellata*, com formação de cristais.

Em adição aos novos sistemas de imobilização, levantou-se a hipótese de que a preservação da estrutura do talo propiciaria a manutenção do contato entre os simbiontes, promovendo uma maior produtividade pelas células imobilizadas.

3 CARACTERIZAÇÃO MORFOLÓGICA E QUÍMICA DA ESPÉCIE ESTUDADA

Canoparmelia texana (Tuck.) Elix & Hale, é um líquen pertencente à família Parmeliaceae.

Diversos sinônimos já foram atribuídos a essa espécie, como por exemplo, *Parmelia texana e Pseudoparmelia texana.*

C. texana possui hábito folhoso, podendo ser destacada de seu substrato com facilidade (Figura 1).

Todas espécies liquênicas possuem uma composição química definida, com variações em substâncias em menores teores; é o que se denomina de raça química. Esse fato se atribui aos fatores ambientais/microclimáticos, aos quais os liquens tendem a se adaptar (HALE-JR., 1983; REYES *et al.*, 1994; NASH III, 1996).

C. texana possui em sua composição, o ácido divaricático (Figura 2) e a atranorina (Figura 3) como principais componentes (WALKER & LINTOTT, 1997).

O ácido divaricático é um depsídeo da série do orcinol e, a atranorina é um para-depsídeo da série do β-orcinol, ou seja, compostos fenólicos cujas unidades estão unidas por uma ligação éster, geralmente nas posições 1 e 4'.

Figura 1 – *Canoparmelia texana* (Tuck.) Elix & Hale

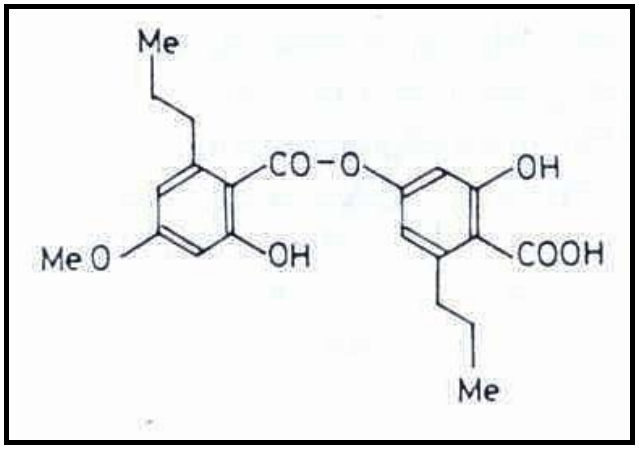

Figura 2 – Fórmula estrutural do ácido divaricático

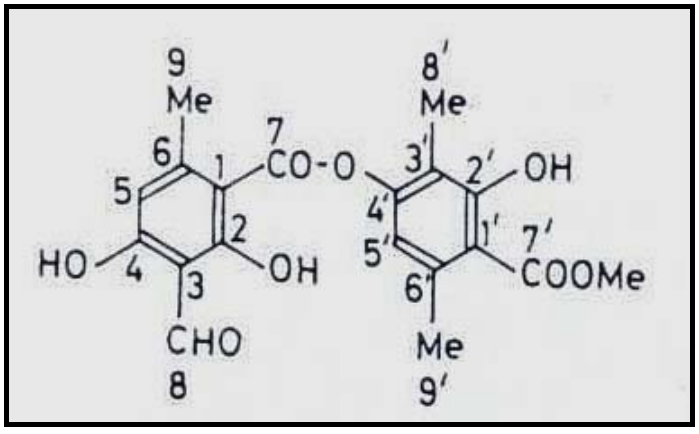

Figura 3 – Fórmula estrutural da atranorina

4 JUSTIFICATIVA E RELEVÂNCIA

Os compostos fenólicos produzidos por liquens são exclusivos deste táxon, uma vez que não há registros dos referidos compostos em representantes do reino Plantae (CULBERSON *et al*., 1977).

Pouco se conhece a respeito das enzimas que sintetizam ou catabolizam tais substâncias e, menos ainda, sobre sua regulação metabólica. Além disso, possuem aplicações diversas e potencialmente interessantes. Há séculos são empregados como fixadores em perfumaria, e por muito tempo utilizadas como potentes antibióticos de uso tópico (VARTIA, 1973). Sua alta atividade citotóxica, um inconveniente para seu uso terapêutico, está sendo atualmente investigado objetivando seu uso como carcinostático (KUMAR & MULLER, 1999).

Em cosmética terapêutica, vários compostos fenólicos de liquens são utilizados para evitar o envelhecimento da pele devido à idade, ou a prolongadas exposições ao sol. A elastase, assim como a tripsina, são fortemente inibidas por atranorina, um fenol de origem liquênica (PROKSA *et al*., 1994).

Não deve ser negligenciada a necessidade de investigação da imensa flora do Brasil e do resto do mundo, visando a busca de novas drogas que trariam benefícios para todos (MONTE, 1998). Portanto, é de grande relevância o estudo do potencial que os liquens podem oferecer, como também a viabilidade da bioprodução de seus compostos, o que evitaria a destruição da micota liquenizada.

5 OBJETIVOS

5.1 Geral

Estudar de forma qualitativa os metabólitos produzidos por células e talo *in natura* de *Canoparmelia texana* imobilizados em caulinita, utilizando como precursor o acetato de sódio, em sistema fixo, em movimento e sob fluxo contínuo, comparando-os com o material isolado do talo *in natura*.

5.2 Específicos

Imobilizar células e fragmentos do talo *in natura* de *C. texana* por diferentes sistemas, para bioprodução de metabólitos.

Testar a eficiência da caulinita como matriz de enclausuramento para esta espécie.

Determinar o sistema e a concentração do precursor que proporcionam maior produtividade.

Determinar o período de vitalidade das células nos diferentes sistemas de imobilização.

Correlacionar a produção de metabólitos na natureza com a de células e dos fragmentos de talo *in natura* imobilizados em laboratório pelos três processos.

6 REFERÊNCIAS BIBLIOGRÁFICAS

ABRAHAN, E. P.; FLOREY, H. W. Antimicrobial substances from lichens and algae. In: *Antibiotic.* V. 1, cap. 13. p.566-575, 1949.

ADAMO, P.; GIORDANO, S.; VINGIANI, S.; *et al.* Trace element accumulation by moss and lichen exposed in bags in the city of Naples (Italy). **Environmental Pollution.** v. 122. p. 91-103, 2003.

ALBUQUERQUE, U. P.; ATANÁZIO, V.; SILVA, N. H.; *et al.* Produção de metabólitos por *Pseudocyphellaria aurata* a partir de imobilização celular. In: **51o Congresso Nacional de Botânica**, Brasília, DF, p. 21, 2000.

COSTA-FILHO, L.; OLIVEIRA, A. F. M.; BRASILEIRO, V. L. F.; *et al.* Contribuição ao estudo do controle biológico de *Dysdercus maurus*, através de substâncias liquênicas. **Resumos do IV Congresso Nordestino de Ecologia**, Sociedade Nordestina de Ecologia, Recife-PE, p. 36, 1991.

CULBERSON, C. F.; CULBERSON, W. L.; JOHSON, A. Second supplement to chemical and botanical guide of lichen products. St. Louis, **The American Bylogical and Lichenological Society,** Inc., 1977. 400p.

CULBERSON, C. F.; AHMADJIAN, V. Artificial reestablishment of lichens II. Secondary products of resynthesed *Cladonia cristatella* and *Lecanora chrysoleuca*. **Mycologia**, v. 72, p. 90-109, 1980.

FONTANIELLA, B.; LEGAZ, M. E.; PEREIRA, E. C.; *et al*. Requirements to produce fumarprotocetraric acid using alginate-immobilized cells of *Cladonia verticillaris.* **Biotechnol Lett.**, v. 22, p. 813-817, 2000.

GARCIA-JUNCEDA, E.; VICENTE, C. The use of immobilized cells to stabilize orsellinate depside hydrolase of *Pseudevernia furfuracea*. **Plant Cell Reports**, v. 5, p. 155-157, 1986.

GBEWONYO, K.; MEIER, J.; WANG, D. I. C. Immobilization of mycelial cells on celite. **Methods in Enzimology,** New York**,** v. 135, p. 318-333, 1987.

GONZALEZ, A.; VICENTE, C.; LEGAZ, M. E. A simple assay demonstrating the effect of rehydration on the orsellinate despide hidrolase activity of *Evernia prunastri*. **J. Plant Physiol.**, v. 116, p.
219-224, 1984.

GUIDOTTI, M.; STELL, D.; OWCZAREK, M.; *et al*. Lichens as polycyclic bioaccumulators used in atmospheric pollution studies. **Journal of Chromatography,** v. 985. p. 185-190, 2003.

HALE-JR., M. E. **The Biology of Lichens.** 3ed. London. Edward Arnold pub., 1983, 90p.

HONDA, N. K.; VILEGAS, W. A química de liquens. **Química Nova.** V. 22(1). p. 25-55, 1999.

HONDA, N. K. A natureza das substâncias produzidas por liquens. In: XAVIER-FILHO, L.; LEGAZ, M. E.; CORDOBA, C.V. ; PEREIRA, E. C. **Biologia de liquens.** Rio de Janeiro: Âmbito Cultural, p. 345-388, 2006.

KUMAR, S., MULLER, K. Lichens metabolites, 2, Antiproliferative and cytotoxic activity of gyrophoric, usnic, and diffractaic acids on human keratinocyte growth. **J. Nat. Products,** 62: 821-823, 1999.

LAWREY, J. D. Inhibition of moss spore germination by acetone extracts of terricolous *Cladonia* species. **Bull. Torrey Bot. Club**., v.104, n.1, p. 49-52, 1977.

LEINZ, V.; AMARAL, S. E. **Geologia Geral.** São Paulo: Companhia Editora Nacional. 1978. 400p.

LIMA, M. J. G. **Produção do ácido úsnico de *Cladonia substellata* Vainio (líquen) por imobilização celular, utilizando diferentes métodos**. Dissertação (Mestrado em Bioquímica) – Departamento de Bioquímica da Universidade Federal de Pernambuco. Recife, 2004.

LIMA, R. M. C.; NASCIMENTO, S. C.; PEREIRA, E. C.; *et al*. Atividade citotóxica e antitumoral de extratos liquênicos. **Bol. Soc. Brot.** V. 63. p. 339-348, 1990.

LLANO, G. A. Economic uses of lichens. **Smithsonian Institution Publ**., v. 4040, p.385-422, 1951.

MACFARLANE, J. D.; KERSHAW, K. A. Some aspects of carbohydrate metabolism in lichens. IN: **Lichen Physiology and Cell Biology.** Ed. D. H. Brown. Plenum Press. N. York, p. 1-8. 1984.

MAZZITELLI, S. M. A. M.; MOTA-FILHO, F. O.; PEREIRA, E. C.; *et al.* Utilização de liquens no biomonitoramento da qualidade do ar. In: XAVIER-FILHO, L.; LEGAZ, M. E.; CORDOBA, C.V. ; PEREIRA, E. C. **Biologia de liquens**. Rio de Janeiro: Âmbito Cultural, p.101-143, 2006.

MONTE, F. J. Q. Produtos naturais vale investigar. **Jornal da Ciência,** Rio de Janeiro. v. 387. p. 4-5, 1998.

MOSBACH, K.; MOSBACH, R. Entrapment of enzymes and microorganisms in synthetic crosslinked polymers and their application in column techniques. **Acta Chem. Scand.**, v. 20, p. 2807-2810, 1966.

MOSBACH, K. The potencial in biotechnology of immobilized multistep enzyme-coenzyme systems. **Phil. Trans. R. Soc.** London, v. 330, p. 355-367, 1983.

NASH III, T. H. **Lichen Biology.** Cambridge, USA, Cambridge, University Press. led. 1996. 303p.

NÓBREGA, N. A. **Produção de compostos fenólicos por células imobilizadas do líquen *Parmotrema andinum* (Müll. Arg.) Hale**. Dissertação (Mestrado em Bioquímica) – Departamento de Bioquímica da Universidade Federal de Pernambuco. Recife, 2002.

PEREIRA, E. C. **Influência da sazonalidade na detecção de atividade antimicrobiana de *Cladonia* e *Cladina* (líquen)**. 193 f. Dissertação. (Curso de Mestrado em Criptógamos). Departamento de Micologia, Departamento de Botânica. Universidade Federal de Pernambuco, 1989.

PEREIRA, E. C.; NASCIMENTO, S. C.; LIMA, R. M. C.; *et al*. Analysis of *Usnea fasciata* crude extracts with antineoplasic activity. **Tokai. J. of Exp. And Clin. Medicine.** V. 19(12). P. 47-52, 1994a.

PEREIRA, E. C.; MOLINA, M. C.; PEDROSA, M. M. *et al.* Production of ribitol by alginate immobilized cells of the lichen *Cladonia verticillaris*. **Anales de Química**, Madrid, v. 91, n. ¾ , p. 253-259, 1995a.

PEREIRA, E. C.; PEREYRA, T.; MATOS, S. C. *et al*. Bioproduction of usnic acid from acetate by kaolinite immobilized cells of *Cladonia substellata* Vanio. **Acta Societatis Botanicorum Poloniae**, Warszawa, v. 64, n. 2, p.171-174, 1995b.

PEREIRA, E. C.; SILVA, N. H.; BRITO, E. S. A.; *et al*. Atividade antimicrobiana de liquens amazônicos. **Rev. U. A.**, Série Ciências Biológicas, v.1, n.1, p. 65-77, 1996.

PEREIRA, E. C. **Produção de metabólitos por espécies de Cladoniaceae a partir de imobilização celular.** 1998. 240f. Tese (Doutorado em Botânica) – Universidade Federal Rural de Pernambuco, UFRPE, 1998.

PEREIRA, E. C.; SILVA, E. F.; SILVA, N. H.; *et al.* Produção de metabólitos de C*ladina corallifera* (Kunze) Nyl. Por imobilização celular. **Revista da Universidade do Amazonas,** Série de Ciências Biológicas, v. 2, n. ½, 1999a.

PEREIRA, E. C.; SILVA, N. H.; ANDRADE, L. C.; *et al.* Production of lichen metabolites by immobilized cells of *Cladonia clathrata*, **Phyton (Austria)**, v. 39, p. 79-89, 1999b.

PEREIRA, E. C.; ANDRADE, L. C.; SILVA, N. H.; *et al*. Production of metabolites by immobilized cells of *Cladia aggregata* (Sw.) Nyl. At different status of fertility. In: **Lichenology in Latin America II**. S. Calvelo & T. Feuere (Eds.). Hamburg, Alemanha, 2002, v. 1, p. 157-169.

PROSKA, B.; ADAMCOVA, J.; STURDIKOVA, M.; *et al.* Metabolites of *Pseudevernia furfuracea* and their inhibition potential of proteolytic enzymes. **Pharmazie**, v. 49, p. 282-283, 1994.

REYES, A.; MOLINA, M. C.; VICENTE, C.; *et al*. Influência da variação ambiental na composição fenólica de diferentes espécies de liquens. **Resumos do II Congresso de Ecologia do Brasil** Londrina-PR, v. 1, p. 22, 1994.

ROCHA, J. A. M. R. **Análise de metabólitos de *Heterodermia leucomela* (L.) Poelt produzidos pelo talo *in natura* e por células imobilizadas**. Recife, 1999. 54p. (Dissertação-Mestrado em Bioquímica-UFPE).

SANTOS, R. A.; PEREIRA, E. C.; SILVA, N. H.; *et al.* Bioprodução de metabólitos de *Ramalina aspera* (líquen) a partir de imobilização celular. In: **VIII Congresso de Iniciação Científica da UFPE-CONIC**, Recife-PE, p. 67, 2000.

SKRYABIN, G. K.; KOSHCHEEHKO, K. A. Immobilization of living microbial cells in polyacrilamide gel. **Methods in Enzymology**, New York, v. 135, p. 198-216, 1987.

SVITEL, J.; CURILLA, O.; TKAC, J. Microbial cell-based biosensor for sensing glucose, sucrose or lactose. **Biotechnology Applied Biochemistry**, London, v. 27, p. 153-158, 1998.

VARTIA, K. O. Antibiotics in lichens. In: **The Lichens** (AHMADJIAN, V., HALE, M. E., eds.), Academic Press, New York, p. 547-561, 1973.

VICENTE, C. **Fisiologia de las substâncias liquénicas.** Alhambra, Madrid. 1975.

VICENTE, C.; MOLINA, M. C. Enzymatic degradation of physodic acid and parietin by stored thalli of *Pseudevernia furfuracea* and *Xanthoria parietina* or by immobilized enzymes and cells. **Bibl. Lichenol.**, v. 53, p. 267-276, 1993.

VICENTE, C.; SOLAS, M. T.; PEREYRA, M. T.; *et al.* Immobilization of lichen cells and enzymes for bioproduction of lichen metabolites: technical requirements and optimization of product recovering. In: **Flechten Follmann. contributions to lichenology in honour of Gerhard Follmann.** J. A. DANIELS; M. SCHULZ; J. PEINE (Eds.). The Geobotanical and Phytotaxonomical Study Group, Botanical Institute, University of Cologne, Germany, p. 97-110, 1995.

WALKER, J. R. L.; LINTOTT, E. A. A phytochemical register of New Zealand lichens. **New Zealand Journal of Botany.** v. 35. p.369-384, 1997.

WALKER, T. R.; CRITTENDEN, P. D.; YOUNG, S. D. Regional variation in the chemical composition of winter snow pack and terricolous lichens in relation to sources of acid emissions in the USA river basin northeast European Russia. **Environmental Pollution.** v. 125 (3). p. 401- 412, 2003.

YAMAMOTO, K.; TOSA, T.; TAMASHITA, K.; *et al*. Continuous production of L-malic acid by immobilizies *Brevibacterium* ammoniagenes cells. **European Journal Applied Microbiology,** New York. v. 3. p. 169-183, 1976.

YANO, A. M. **Atividade biológica de *Cladonia verticillaris* e *Cladonia substellata* sobre a germinação e desenvolvimento da plântula de *Allium cepa.*** 126 f. Dissertação. (Curso de Mestrado em Criptógamos). Departamento de Micologia, Departamento de Botânica, Universidade Federal de Pernambuco, 1994.

YOSHIMURA, I.; KUROKAWA, T.; YAMAMOTO, Y.; *et al*. Development of lichen thalli *in vitro*. **Bryologist**, v. 96, p. 412-421, 1993.

7. ARTIGOS A SEREM SUBMETIDOS À PUBLICAÇÃO

7.1 Capítulo I

BIOPRODUCTION OF PHENOLIC COMPOUNDS METABOLITES FROM *Canoparmelia texana* (LICHEN) BY USING IMMOBILIZED CELLS

Biotechnology Letters

Bioproduction of Phenolic compounds Metabolites from *Canoparmelia texana* (lichen) by using immobilized cells

Serafim, A. T. N. [1]; Pereira, E. C.[2 *] ; Lima, M. J. G. [1]; Eliasaro, S.[3] & Silva, N. H.[1]
[1]Departamento de Bioquímica, Universidade Federal de Pernambuco, Recife, Brasil
[2]Departamento de Ciências Geográficas, Universidade Federal de Pernambuco, Recife, Brasil
[3]Departamento de Botânica, Universidade Federal do Paraná, Curitiba, Brasil
**Corresponding author: (Fax: 55-81-2126.8275) E-mail: eugenia.pereira@pesquisador.cnpq.br*

Key words: lichen, divaricatic acid, atranorin, *Canoparmelia texana,* cellular immobilization

Abstract

Bioproduction of phenolic compounds from *Canoparmelia texana* (lichen) immobilized cells in different systems: fixed or traditional, moving and under continuous-flow, using sodium acetate as precursor and kaolinite as entrapment matrix, resulting in a promising biotechnology for the production of atranorin and divaricatic acid.

Introduction

The interest in lichen substances has been growing strongly among the scientific community that works with the pharmacology of natural products, once their therapeutic effects are evident (Hale-Jr., 1983).

For centuries, the lichen metabolites have been commercially exploited, as fixers in perfumery or as potent topical antibiotics (Vijayakumar *et al.*, 2000).

The atranorin, a phenol of lichen origins, is used in therapeutic cosmetics because it promotes a strong inhibition of elastase and trypsin (Rancan *et al.*, 2002) presenting, also, anti-inflammatory activity (Maia *et al.,* 2002).

Currently, due to the high cytotoxicity of some substances, which is a disadvantage for therapeutic use, the possibility of using these substances as carcinostatic is under investigation (Santos *et al.*, 2003).

In the presence of a diversity of uses from lichen metabolites in pharmaceutical, cosmetic and food industries, its application at commercial level must be cautious.That is because of the need for a destruction of a great amount of biomass, hardly renewable, to obtain a compound of economic interest (Vicente *et al.,*1995).

An alternative to product lichen substances for industrial and scientific purposes, without destroying the lichen mycota, is the use of cellular immobilization. In this approach cells are captured in a defined space, with their activity totally or partially preserved for repeated and

continued use. It is an attractive alternative to the use of purified enzymatic systems, since the cell itself organizes and provides the ideal operation conditions (Svitel e*t al.*, 1998).

Material and methods

Lichen material

Canoparmelia texana (Tuck.) Elix & Hale lichen, collected in the city of Curitiba – PR/Brazil, was used during the development of this experiment.

Collection and storage

About 200g of *C. texana* were stored in paper bags and kept at room temperature (28ºC ± 3ºC) until the experiments were carried out.

The material was identified by one of the authors (S. Eliasaro) and part of the sample was deposited at UFP Herbarium, from Universidade Federal de Pernambuco, Brazil, voucher nº 44627.

Obtention of organic extracts from thallus in natura

From the dry thallus at room temperature (28°C ± 3°C), organic extracts of *C. texana* were obtained with the use of diethyl ether, chloroform and acetone through a exhaust system, the hot, obeying the solvents eluotropic series.

Cellular immobilization in a fixed system

A sample (2g) of *C. texana* as used in each attempt at cellular immobilization, according to Pereira *et al*. (1995).

The cells were immobilized in 90g of kaolinite previously hydrated for 2 h, with deionized water. The disabled material was grouped in columns and to each one, 30mL of sodium acetate in 0.1, 1.0 and 10.0 mM concentrations were added. The columns were kept under white light (125 $\mu mol.m^{-2}.s^{-1}$) for 2 months. Periodically, 30 mL samples from each column were collected and a fresh solution of sodium acetate was re-added in the respective concentration and volume.

Cellular immobilization in a moving system

The system was prepared in the same way as the fixed system, although the immobilized material was set in test-tubes and, to each one, 30 mL of sodium acetate were added under the concentrations above mentioned. The test-tubes were fixed in a pulley attached to an engine that kept it rotating at a steady speed during all the experiment. Aliquots 30 mL were collected and reset in their volume in the respective concentration of precursor.

Cellular immobilization in a continuous-flow system

Cells disabled in kaolinite, as described above, were placed in a porous funnel and again added to 30 mL of the precursor under the concentrations referred to in the other methods, through a continuous-dripping system. Fractions 30 mL of the precursor were collected and added continuously during the experiment, through dripping, to the respective concentrations.

Fraction attainment and treatment

Initially, aliquots were collected on a daily basis for the first seven days. Subsequently the collections occurred 15, 30, 45 and 60 days from the beginning of the experiment.

The aliquots collected through each method remained frozen until processing time. A sample (30 mL) of diethyl ether/ethyl acetate (65:35, v/v), as used to obtain the extracts, followed by three stirrings and rest. After separation of phases, the same volume of chloroform/acetonitrile (60:40, v/v) system was added to aqueous phase, proceeding the same way as previous extraction. Both extracts were submitted to spectrophotometer measure at 254nm and 366nm. Thereafter, they were evaporated for subsequent analysis (Pereira *et al*., 1995).

Thin Layer Chromatography

Thin layer chromatography was used to qualify detection of predominant phenolic compounds in the *in natura* thallus organic extracts and cellular eluates of *C. texana* obtained in three immobilization experiments. Diluted samples were applied in silica gel 60 $F_{254 + 366}$ Merck of 20 x 20 cm plates and developed upwardly (ascending) in the solvent system A, consisted of toluene/dioxane/acetic acid (180:45:5, v/v). After evaporation of solvents, bands were visualized under UV short (254nm) and long (366nm) wavelenght. Then, the plates were sprayed with 10% sulphuric acid and heated at 100ºC for 1 h, in order to evidence the bands through coloration reaction (Culberson, 1972). The results were compared to atranorin and divaricatic acid standards.

Results and discussion

The lichen substances are formed by phenolic units that originate in policetonic carboxylic acids, derived from acetic acid that the lichen enzymatic system uses in acetate form.

Therefore, sodium acetate (NaOAc) was used as precursor to the synthesis of phenolic compounds.

The organic extracts obtained fractions collected from bioreactors (fixed, moving and continuous-flow) and submitted to spectrophotometer at 254nm and 366nm, revealed the synthesis of substances by immobilized cells in all immobilization systems and in all concentrations of the precursor (0.1, 1.0 and 10.0 mM), with a greater predominance of substances with polarity similar to the system containing diethyl ether/ethyl acetate, which can be evidenced through the registration of the absorption of both extracts (figures 1, 2, 3).

In the fixed system, the bioproduction in the different concentrations of the precursor showed an initial peak of productivity of the phenolic compounds, with subsequent decrease until the sixth day of the experiment (144h), followed by no production of these compounds at the end of the first week (168h) (figure 1). According to figure 4, the concentration of the precursor added to this immobilization system has practically not influenced the productivity of immobilized cells. A similar fact occurs with *Cladonia corallifera,* since the production of phenolic compounds is practically equal, even when the concentration of precursor is changed (Pereira *et al.*, 2002).

In the moving system (Figure 2), if the lengths wave of both organic extracts are analyzed in all concentrations of precursor, it is shown that the cells remained productive during the experiment, as variations in the productivity of substances were registered, at times at a decreasing and at an increasing rate. The greatest production of phenolic compounds was observed in the concentration of 10.0 mM NaOAc (Figure 4).

In continuous-flow system, the bioproduction occurred through an initial maximum peak followed by a sharp drop in the production. However, despite the small variations during the experiment, the cells practically ceased the production after the first week of experiment (Figure 3). The level of production in this system occurred decreasingly compared to the concentration of precursor, whereas the bioreactor with 0.1 mM of NaOAc was the one that produced the most (Figure 4).

Analyzing all three immobilization systems, the reduction or even the cessation of the production wes observed during the first week of experiment with fixed and continuous-flow systems, as opposed to moving system, in which the production occurs during 2 months, justifying the need for a continuous movement of bioreactor, which provides the contact between precursor and immobilized cells.

This contact is damaged in the other systems once the mixture, in suspension form, containing the immobilized cells in kaolinite and the sodium acetate solution, in time, suffers

decantation promoting the separation between cells and precursor, thus damaging the production of phenolic compounds.

Despite this need, the continuous-flow system was the one that bioproduced the most, in total amounts, especially in the 0.1 mM concentration of NaOAc, because it is possible that the accumulation of bioproduced substances in the environment containing NaOAc causes the cells to use, somehow, these compounds in their metabolisms. The continuous-flow system reduces the contact time of these substances with cells, not saturating the environment, and making impossible for the immobilized cells to reutilize them. That explains a greater productivity of this system, mainly in the first week of experiment, due to reduction in contact between cells and the precursor with the passing of time and/or according to Pereira *et al.* (1999), the contact between symbionts can be damaged, making the transference of enzymes and cofactors important to the biosynthesis of these compounds.

Through TLC of organic extracts of *in natura* thallus the presence of atranorin and divaricatic acid was observed, and so was another not identified compound in the ethereal extract: atranorin and a second not identified compound in the chlorophormic extract, and only the divaricatic acid in the acetonic extract, being in accordance with Walker *et al.* (1997) who cites the divaricatic acid and the atranorin as the main components of the *C. texana* as well as the reports that all lichen species have a definite chemical composition, with variations in substances – generally not identified – at lower rates, which is denominated the chemical race (Hale-Jr., 1983; Nash III, 1996).

To date, it has been evidenced that, in general terms, some species have the production of compounds similar to the ones obtained from the *in natura* thallus, through its immobilized cells; others synthesize intermediary products of their metabolic routes, or their phenols in their reduced form (Pereira *et al.*, 1999). The *in natura* thallus fragments of *C. texana*, immobilized in different systems, bioproduced the same substances contained in the *in natura* thallus (Figure 5), indicating not only the adaptation of studied species to the process of cellular immobilization, but a promising source of biotechnology for the production of atranorin and divaricatic acid, two substances with countless biological applications.

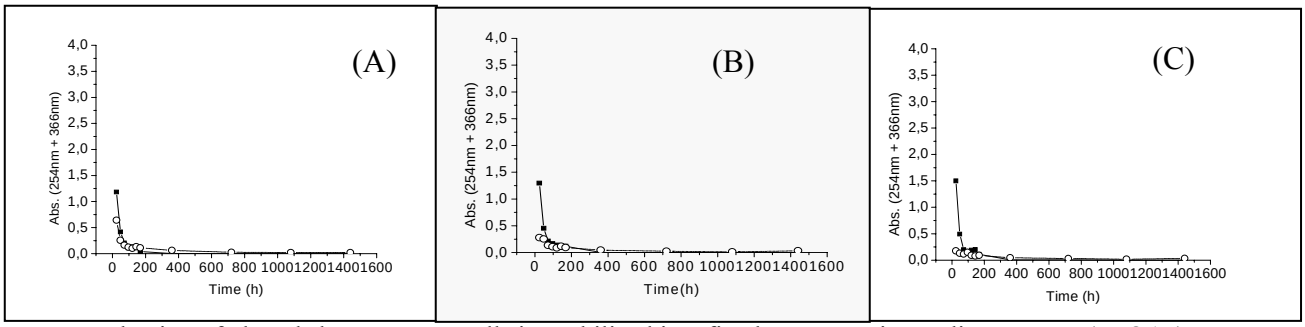

Fig. 1 Production of phenols by *C. texana* cells immobilized in a fixed system, using sodium acetate (NaOAc) as precursor a 0.1 mM (A); 1.0 mM (B) and 10.0 mM (C).-▪- diethyl ether/ethyl acetate/ -○- chloroform/acetonitrile

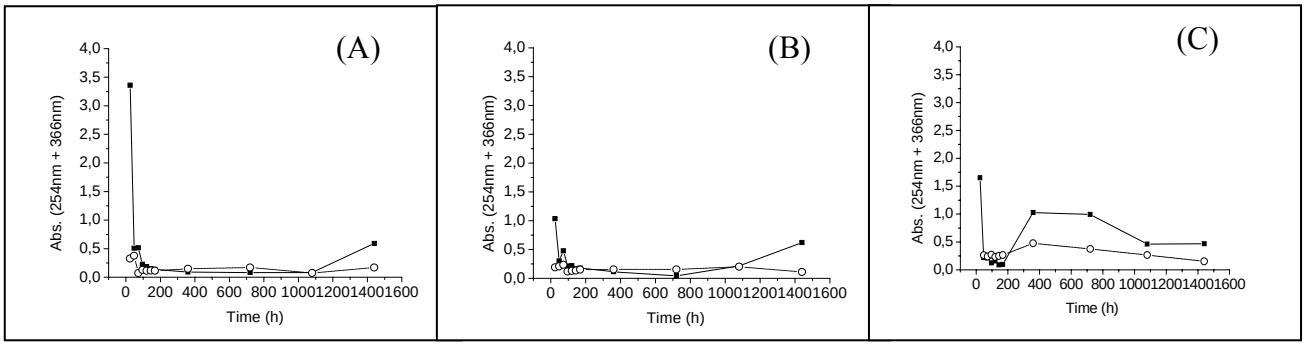

Fig. 2. Production of phenols by *C. texana* cells immobilized in a moving system, using sodium acetate (NaOAc) as precursor a 0.1 mM (A); 1.0 mM (B) and 10.0 mM (C) . -▪- diethyl ether/ethyl acetate / -○- chloroform/acetonitrile

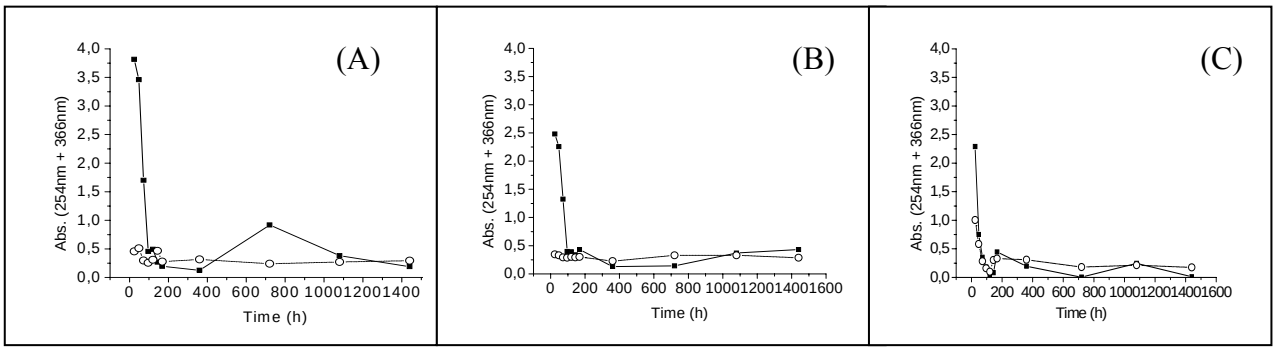

Fig. 3. Production of phenols by *C. texana* cells immobilized in a continuous-flow system, using sodium acetate (NaOAc) as precursor a 0.1mM (A), 1.0 mM (B) and 10.0 mM (C).
-▪- diethyl ether/ethyl acetate / -○- chloroform/acetonitrile

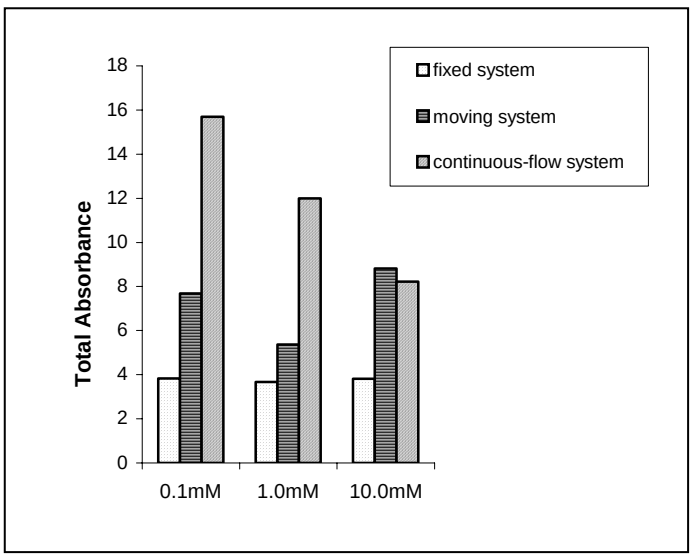

Fig. 4. Total production average of phenolic compounds on three systems of *C. texana* cellular imobilization using NaOAc as precursor at 0.1, 1.0 and 10.0 mM.

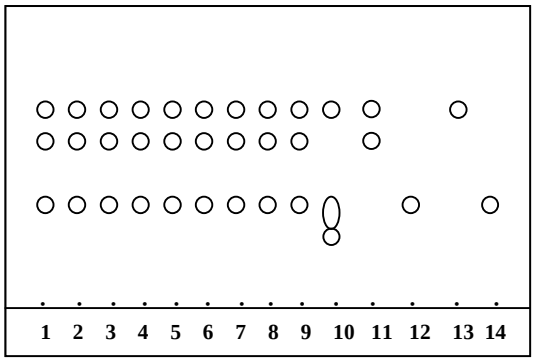

Fig. 5. Thin layer chromatogram of fractions obtained from cellular immobilization in different systems, organic extracts and standard substance of *C. texana*, developed on A solvent system (toluen/ dioxane/acetic acid, 180:45:05, v/v).
Fractions obtained by cellular immobilization :
1. 0.1mM/ in a fixed system 2. 1.0mM/ in a fixed system
3. 10.0mM/ in a fixed system 4. 0.1mM/ in a moving system
5. 1.0mM/ in a moving system 6. 10.0mM/ in a moving system
7. 0.1mM/ in a continuous-flow system 8. 1.0mM/ in a continuous-flow system
9. 10.0mM/ in a continuous-flow system
Organic extracts: 10. Ether 11. Chlorophorm 12. Acetone
Standard substance: 13. Atranorin 14. Divaricatic acid

References

Culberson C F (1972) Improved conditions and new data for the identification of lichen products by standardized thin layer chromatographic method. Journal of Chromatography 72:113-125.

Hale-Jr. M E (1983) The Biology of Lichens. 3rd edn. Edward Arnold pub., London.

Maia M B S, Silva N H, Silva E F *et al.* (2002) Antinoceptive activity of crude extract and atranorin obained from the lichen *Cladina dendroides* (des Abb.) Ahti. Acta Farm. Bonaerense 21(4):259-264.

Nash III T H (1996) Lichen Biology. Cambridge, USA, Cambridge, University Press. led.

Pereira E C, Pereyra T, Matos S C *et al*. (1995) Bioproduction of usnic acid from acetate by kaolinite immobilized cells of *Cladonia substellata* Vanio. Acta Societatis Botanicorum Poloniae, Warszawa 64(2): 171-174.

Pereira E C, Silva N H, Andrade L C *et al.* (1999) Production of lichen metabolites by immobilized cells of *Cladonia clathrata*. Phyton (Austria) 39: 79-89.

Pereira E C, Andrade L C, Silva N H *et al*. (2002) Production of metabolites by immobilized cells of *Cladia aggregata* (Sw.) Nyl. At different status of fertility. In: S. Calvelo & T. Feuere (Eds.) Lichenology in Latin America II. Hamburg, Alemanha.

Rancan F, Rosan S, Boehm K *et al.* (2002) Protection against UVB irradiation by natural filters extracted from lichens. Journal of Photochemistry and Photobilogy B: Biology 68: 133-139.

Santos N P, Wanderley M S O, Pereira E C G *et al.* (2003) In vitro and in vivo antitumoral activity of nanocapsules containing usnic acid. Brazilian Journal of Pharmaceutical Sciences 39(2).

Svitel. J, Curilla O, Tkac J (1998) Microbial cell-based biosensor for sensing glucose, sucrose or lactose. Biotechnology Applied Biochemistry 27:153-158.

Vicente C, Solas M T, Pereyra M T *et al.* (1995) Immobilization of lichen cells and enzymes for bioproduction of lichen metabolites: technical requirements and optimization of product recovering. In: Daniels J A, Schulz M, Peine J. (Eds.) Flechten Follmann. contributions to lichenology in honour of Gerhard Follmann. The Geobotanical and Phytotaxonomical Study Group, Botanical Institute, University of Cologne, Germany, pp. 97-110.

Vijayakumar CS, Viswanathan S, Kannappa-Reddy M, Parvathavarthini S, Kundu SB, and Sukumar E (2000). Anti-inflammatory activity of (+) usnic acid. Fitoterapia 71: 564-566.

Walker J R L, Lintott E A (1997) A phytochemical register of New Zealand lichens. New Zealand Journal of Botany 35:369-384.

7.2 Capítulo II

PRODUCTION OF METABOLITES FROM IMMOBILIZED THALLUS FRAGMENTS OF C*anoparmelia texana* (TUCK.) ELIX & HALE (LICHEN)

Plant Cell Reports

Production of metabolites from immobilized thallus fragments of *Canoparmelia texana* (Tuck.) Elix & Hale (lichen)

Serafim, A. T. N. [1]; Pereira, E. C.[2 *] ; Wessen, C. K[1]; Eliasaro, S.[3] & Silva, N. H.[1]
[1]Departamento de Bioquímica, Universidade Federal de Pernambuco, Recife, Brasil
[2]Departamento de Ciências Geográficas, Universidade Federal de Pernambuco, Recife, Brasil
[3]Departamento de Botânica, Universidade Federal do Paraná, Curitiba, Brasil
**Corresponding author (Fax: 55-81-2126.8275) E-mail: eugenia.pereira@pesquisador.cnpq.br*

Abstract

This study is aimed at producing metabolites of *Canoparmelia texana* (Tuck.) Elix & Hale from immobilization of *in natura* thallus fragments, through the use of bioreactors with fixed (traditional), moving, and under countinuous-flow system. Thallus fragments, were immobilized, in bioreactors using kaolinite as entrapment matrix and sodium acetate at 0.1, 1.0 and 10.0 mM, as biosynthetic precursor of typical substances of the species. Samples taken at different periods of time were extracted with diethyl ether/ethyl acetate (65:35, v/v) and chloroform/acetonitrile (60:40, v/v), and measured by in the spectrophotometer at 254 e 366 nm. After evaporation, the extracts were assessed through thin layer chromatography. The organic extract spectrophotometer measuring revealed the synthesis of substances by thallus immobilized fragments in all immobilization systems and in all concentrations of precursor. There was a quantitative predominance of substances bioproduced by the moving system. These thallus fragments immobilized under different systems bioproduced the same substances found in the *in natura* thallus, indicating not only the adaptation of studied species to the process of cellular immobilization, but a promising biotechnological source to the production of atranorin and divaricatic acid, two substances with countless biological applications.

Key words: lichen, divaricatic acid, atranorin, *Canoparmelia texana,* cellular immobilization.

Introduction

Biotechnological strategics are promising for lichens and their symbiont cultures, besides the production of their metabolites.

Cell immobilization using different entrapment matrix, shows efficient sugar and phenolic production, but the procedures of cell isolation lead to loss of symbiont contact (Pereira *et al.,* 1999), that prejudices the transference of enzymes and cofactors (Fontaniella *et al*, 2000).

Modifications on bioreactors could promote different kind of contact between cells and precursor, optimizing the biosynthesis process; also the production from immobilized cells from *Canoparmelia texana*, using kaolinite entrapment matrix, and sodium acetate as biosynthetic precursor, have already been evaluated (Serafim *et al*., *in press*). They could affirm the species adaptation to immobilization process, and its ability of producing atranorin and divaricatic acid. Nevertheless, additional compounds were identified in cell washes, probably intermediary compounds, or degradation products.

The main objective of this study was to immobilize thallus fragments of *C. texana*, in different bioreactors, for determining the efficiency of modification of cell obtention for phenolic bioproduction.

Material and methods

Lichen material

Canoparmelia texana (Tuck.) Elix & Hale lichen, collected in the city of Curitiba – PR/Brazil, was used during the development of this experiment.

Collection and storage

About 200g of *C. texana* were stored in paper bags and kept at room temperature (28ºC ± 3ºC) until the experiments were carried out.

The material was identified by one of the authors (S. Eliasaro) and part of the sample was deposited at UFP Herbarium, from Universidade Federal de Pernambuco, Brazil, voucher nº 44627.

Obtention of organic extracts from thallus in natura

From the dry thallus at room temperature (28°C ± 3°C), organic extracts of *C. texana* were obtained with the use of diethylic ether, chlorophorm and acetone through a exhaustion system, the hot, obeying the solvents eluotropic series.

Cellular immobilization in a fixed system

A sample (2g) of *C. texana* as used in each attempt at cellular immobilization, according to Pereira *et al*. (1995). However, the thallus *in natura* was fragmented by scissors, not macerated.

The thallus fragments were immobilized in 90g of kaolinite previously hydrated for 2h, with deionized water. The disabled material was grouped in columns and to each one, 30mL of sodium acetate in 0.1, 1.0 and 10.0 mM concentrations were added. The columns were kept under white light (125 $\mu mol.m^{-2}.s^{-1}$) for 2 months. Periodically, 30 mL samples

from each column were collected and the solution of sodium acetate was re-added in the respective concentration and volume.

Cellular immobilization in a moving system

The system was prepared the same way as the fixed system, although the immobilized material was set in test-tubes and, to each one, 30 mL of sodium acetate were added under the concentrations above mentioned. The test-tubes were fixed in a pulley attached to an engine that kept it rotating at a steady speed during all the experiment. Aliquots 30 mL were collected and reset in their volume in the respective concentration of precursor.

Cellular immobilization in a continuous-flow system

Thallus fragments disabled in kaolitine, as described previously, were placed in a porous funnel and again added to 30 mL of the precursor under the concentrations referred to in other methods, through a continuous-dripping system. Fractions 30 mL of the precursor were collected and added continuously during the experiment, through dripping, to the respective concentrations.

Fraction attainment and treatment

Initially, aliquots were collected on a daily basis for the first seven days. Subsequently the collections occurred 15, 30, 45 and 60 days from the beginning of the experiment.

The aliquots collected through each method remained frozen until processing time. A sample (30 mL) of diethyl ether/ethyl acetate (65:35, v/v), as used to obtain the extracts, followed by three stirrings and rest. After separation of phases, the same volume of chloroform/acetonitrile (60:40, v/v) was added to aqueous phase, proceeding the in same way as previous extraction. Both extracts were submitted to spectrophotometer measuring at 254nm and 366nm. Thereafter, they were evaporated for subsequent analysis (Pereira *et al*., 1995).

Thin Layer Chromatography (TLC)

Thin layer chromatography was used for detecting of predominant phenolic compounds in the *in natura* thallus organic extracts and cellular eluates of *C. texana* obtained in three immobilization experiments. Diluted samples were applied to Gel 60 $F_{254 + 366}$ Merck de 20 x 20 cm plates and developed upwardly (ascending) in the solvent system A, consisted of toluene/dioxane/acetice acid (180:45:5, v/v). After evaporation of solvents, bands were visualized under UVshort (254nm) and long (366nm) wave lenght. Then, the plates were sprayed with sulphuric acid at 10% and heated at 100ºC for 1 h, in order to evidence the bands

through coloration reaction (Culberson, 1972). The results were compared to atranorin and divaricatic acid standards.

Results and discussion

The majority of the secondary metabolites produced by lichens come from the acetate-polimalonate pathway. The acetic acid, in ester form with the co-enzyme A (acethylSCoA), that the lichen enzymatic system uses in the form of acetate, is the basic unit of this biosynthetic route, which leads to formation of aromatic substances (Honda, 2006).

Therefore, sodium acetate (NaOAc) was used as precursor to the synthesis of the phenolic compounds.

The organic extracts obtained in fractions collected from bioreactors (fixed, moving and under continuous flow) and submitted to spectrophotometer measuring at 254nm and 366nm, revealed the synthesis of substances by immobilized cells in all immobilization systems and in all concentrations of precursor (0.1; 1.0 e 10.0 mM), with a greater predominance of substances with similar polarity to the system containing diethyl ether/ethyl acetate, which can be evidenced through the registration of the absorption of both extracts (figures 1, 2, 3).

Figure 1 indicates, in the fixed system, that the phenolic compounds are produced in different concentrations of precursor (0.1, 1.0 e 10.0 mM of NaOAc), during all the experiment. However, it showed the highest peaks of productivity in the first week of immobilization (168h) remaining constant, with subsequent decrease, suggesting the need for bioreactor movement that will support the production of these metabolites due to the promotion of contact between the lichen cells and the precursor.

The 1.0 and 10.0 mM concentrations of NaOAc were the most productive ones in this system (Figure 4).

In moving system (Figure 2), if the wave lengths of both organic extracts are analyzed, in all concentrations of the precursor, it is shown that the cells remained productive during the experiment, since variations in the productivity of substances were registered, at times decreasing and, at times increasing. The highest peaks of production of substances occurred from the first month of immobilization, reaffirming the vitality of cells and, justifying the use of *in natura* thallus and the maintenance of environment under constant movement.

According to Pereira *et al.* (1999) and Fontaniella *et al.* (2000) the separation of cells of micobiont and the photobiont, in the usual cell immobilization systems, leads to the

damage of contact among the symbionts, making it difficult to transfer important enzymes and co-factors for the biosynthesis of these metabolites.

The use of fragments of thallus *in natura* promotes, in addition to this transference of cofactors and enzymes, greater protection of lichen cells from the agreding external agents, thus increasing its vitality.

The maintenance of means in constant movement is fundamental to impede a possible distancing among the immobilized cells and the precursor, since the mixture in suspension form, containing thallus fragments immobilized in kaolitine and the acetate solution, as time elapses, suffers decantation, damaging and even ceasing the production of phenolic compounds.

The production of phenolic compounds practically did not vary with the different concentrations of the precursor (Figure 4).

In the continuous flow system, the bioproduction took place through a maximum initial peak with subsequent drop in the production. At the end of the experiment the cells practically ceased their production (Figure 3). However, when one observes the total productivity of this system (Figure 4), the amount of produced substance is only slightly inferior to the production in the moving system, alerting to the possibility that the accumulation of bioproduced substances in a medium containing NaOAc will cause the cells to use, in some way, these compounds in their metabolism. The continuous flow system reduces the contact time of these substances with the cells, not saturating the medium, thus making its reuse impossible by the immobilized cells. That explains the high productivity of this system, even reducing significantly its production in time, coming practically to zero due to the reduction of contact between cells and the precursor with the passing of time.

The 0.1 mM concentration of NaOAc seems discretely more productive as compared to others (Figure 4) in this immobilization system.

Through TLC of organic extracts of *in natura* thallus the presence of atranorin and divaricatic acid was observed, and so was another not identified compound in the ethereal extract, atranorin and a second not identified compound in the chlorophormic extract, and only the divaricatic acid in the acetonic extract, being in accordance with Walker *et al.*(1997) who cites the divaricatic acid and the atranorin as the main components of the *C. texana* as well as the reports that all lichen species have a definite chemical composition, with variations in substances – generally not identified – at lower rates, which is denominated the chemical race (Hale-Jr., 1983; Nash III, 1996).

Moreover, Serafim *et al.* (*in press*) have obtained similar results when they immobilized cells, extracted from the natural thallus of the *C. texana.* On the other hand, the immobilizations carried out with fragments of thallus have resulted in much greater amounts of bioproduced compounds, as compared to the experiments carried out with isolated cells.

Therefore, in the case of *C. texana*, the use of thallus fragments has promoted greater productivity of substances identical to the *in natura* thallus ones as well as to the immobilized cell ones.

To date, it has been evidenced that, in general terms, some species have the production of compounds similar to the ones obtained from the *in natura* thalus, through its immobilized cells; others synthesize intermediate products of their metabolic routes, or their phenols in their reduced form (Pereira *et al.*, 1999). The *in natura* tallus fragments of *C.texana*, immobilized in the different systems, bioproduced the same substances contained in the *in natura* thallus (Figure 5), indicating not only the adaptation of the studied species to the process of cellular immobilization, but a promising source of biotechnology for the production of atranorin and divaricatic acid, two substances with countless biological applications.

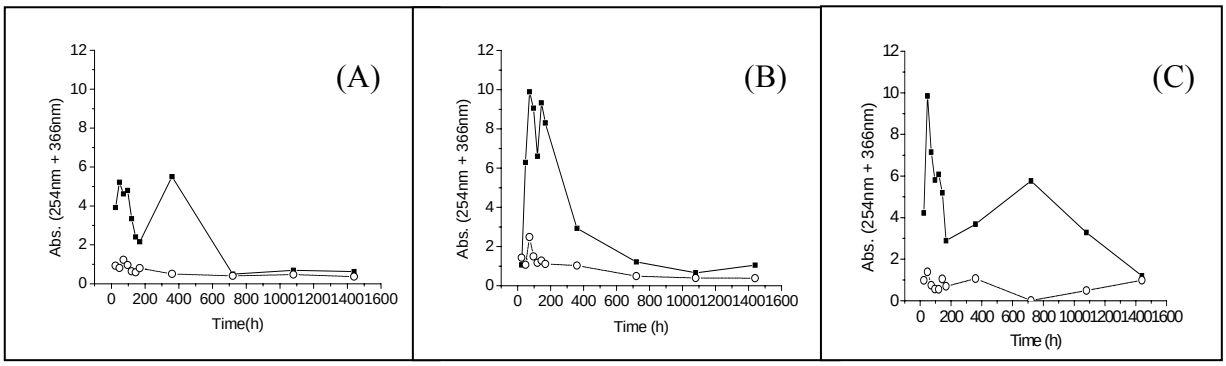

Fig. 1 Production of phenols by *C. texana* fragments of thallus immobilized in a fixed system, using sodium acetate (NaOAc) as precursor a 0.1mM (A); 1.0mM (B) and 10.0mM (C).
-▪- diethyl ether/ethyl acetate / -○- chloroform/acetonitrile

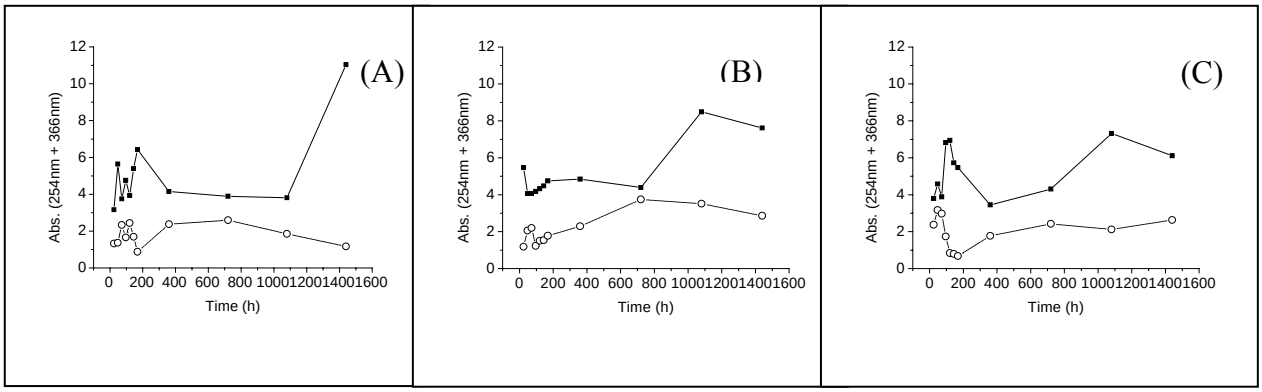

Fig. 2. Production of phenols by *C. texana* fragments of thallus immobilized in a moving systen, using sodium acetate (NaOAc) as precursor a 0.1mM (A); 1.0mM (B) and 10.0mM (C) .
-▪- diethyl ether/ethyl acetate / -○- chloroform/acetonitrile

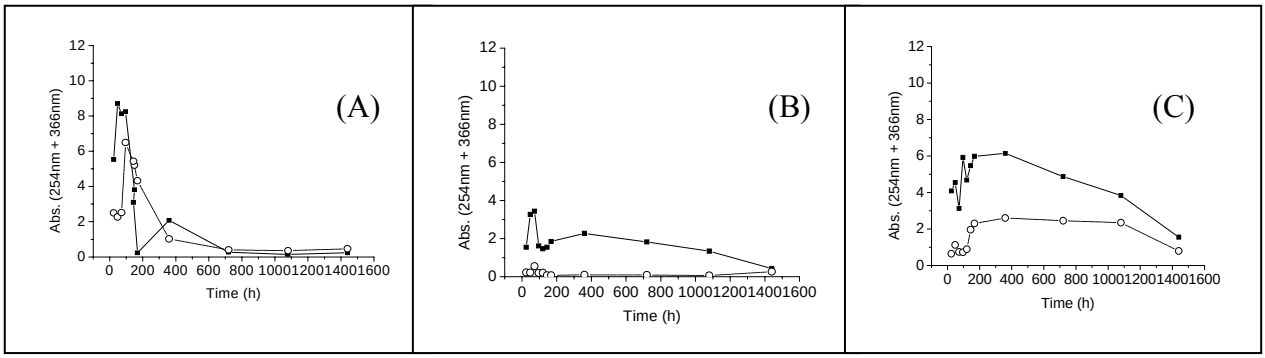

Fig. 3. Production of phenols by *C. texana* fragments of thallus immobilized in a continuous-flow system , using sodium acetate (NaOAc) as precursor a 0.1mM (A); 1.0mM (B) and 10.0mM (C).
-▪- diethyl ether/ethyl acetate / -○- chloroform/acetonitrile

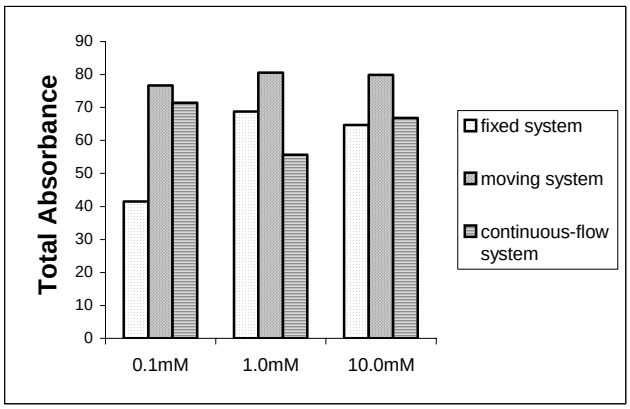

Fig. 4. Total production average of phenolic compounds on three system of *C. texana* cellular imobilization using NaOAc as precursor at 0.1, 1.0 and 10.0 mM.

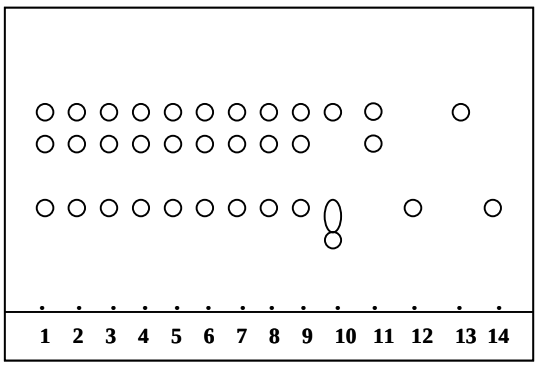

Fig. 5. Thin layer chromatogram of fractions obtained by cellular immobilization in different systems, organic extracts and standard substance of *C. texana*, developed on A solvent system (toluen/ dioxane/acetic acid, 180:45:05, v/v).

Fractions obtained by cellular immobilization :
1. 0.1mM/ System in repose 2. 1.0mM/ System in repose
3. 10.0mM/ System in repose 4. 0.1mM/System in movement
5. 1.0mM/ System in movement 6. 10.0mM/ System in movement
7. 0.1mM/Continuous flux 8. 1.0mM/ Continuous flux
9. 10.0mM/ Continuous flux
Organic extracts: 10. Ether 11. Chlorophorm 12. Acetone
Standard substance: 13. Atranorin 14. Divaricatic acid

References

Culberson C F (1972) Improved conditions and new data for the identification of lichen products by standardized thin layer chromatographic method. Journal of Chromatography, Amsterdam 72:113-125.

Fontaniella B, Legaz M E, Pereira E C *et al* (2000) Requirements to produce fumarprotocetraric acid using alginate-immobilized cells of *Cladonia verticillaris.* Biotechnol Lett 22: 813-817.

Hale-Jr. M E (1983) The Biology of Lichens. 3rd edn. Edward Arnold pub., London.

Honda N K (2006) A natureza das substâncias produzidas por liquens. In: Xavier-Filho L, Legaz M E, Cordoba C V *et al.* Biologia de liquens. Âmbito Cultural, Rio de Janeiro, pp. 345-388.

Nash III T H (1996) Lichen Biology. Cambridge, USA, Cambridge, University Press. led.

Pereira E C, Pereyra T, Matos S C *et al*. (1995) Bioproduction of usnic acid from acetate by kaolinite immobilized cells of *Cladonia substellata* Vanio. Acta Societatis Botanicorum Poloniae, Warszawa 64(2): 171-174.

Pereira E C, Silva N H, Andrade L C *et al.* (1999) Production of lichen metabolites by immobilized cells of *Cladonia clathrata*. Phyton (Austria) 39: 79-89.

Serafim A T N, Pereira E C, Lima M J G *et al.* (*in press*) Bioproduction of Phenolic compounds Metabolites from *Canoparmelia texana* (lichen) by using immobilized cells Biotechnology Letters (*in press*).

Walker J R L, Lintott E A (1997) A phytochemical register of New Zealand lichens. New Zealand Journal of Botany 35:369-384.

8. CONCLUSÕES GERAIS

- A caulinita mostrou-se eficiente como matriz de enclausuramento para a espécie em estudo.
- Na imobilização contendo o filtrado celular, o sistema sob fluxo contínuo obteve o melhor desempenho entre os demais e a concentração de 0,1mM, do precursor, a que mais se destacou na bioprodução dos compostos fenólicos.
- Na imobilização contendo o filtrado celular, as células liquênicas imobilizadas nos diversos sistemas aparentemente não permanecem viáveis por muito tempo, perdendo sua capacidade de bioprodução após a primeira semana de experimento.
- Na imobilização contendo os fragmentos do talo *in natura* os três sistemas e as três concentrações do precursor se mostraram eficientes na bioprodução, com um discreto destaque para o sistema em movimento.
- Na imobilização contendo os fragmentos do talo, as células liquênicas imobilizadas nos diversos sistemas mantiveram sua vitalidade por todo o experimento.
- A imobilização contendo os fragmentos de talo *in natura* mostrou-se mais eficaz, em relação àquela contendo o filtrado celular, apresentando um maior índice de produtividade e uma maior vitalidade das células durante o experimento.
- De acordo com os resultados obtidos a partir de cromatografia em camada delgada, são bioproduzidos compostos fenólicos semelhantes aos encontrados no talo *in natura,* em especial a atranorina e o ácido divaricático.

9. ANEXOS

9.1 Resumos publicados em anais de congressos

9.1.1 Resumo enviado ao VII Encontro do Grupo Latino Americano de Liquenólogos (GLAL) – Curitiba, PR – Novembro, 2005

BIOPRODUÇÃO DE METABÓLITOS DE *Canoparmelia texana* (TUCK.) ELIX & HALE A PARTIR DE IMOBILIZAÇÃO CELULAR*

Serafim, A. T. N.[1]; Lima, C. R. V.[2]; Silva, N. H.[3]; Eliasaro, S.[4]; Pereira, E. C. G.[5]
[1] albaserafim@bol.com.br, Curso de Mestrado em Bioquímica; [2]Graduanda em Bel. Ciências Biológicas/UFPE – Bolsista IC/CNPq; [3]Departamento de Bioquímica/UFPE; [4]Departamento de Botânica/UFPR; [5]Departamento de Ciências Geográficas/UFPE.

Este trabalho teve como objetivo produzir os metabólitos de *Canoparmelia texana* (Tuck.) Elix & Hale através do uso de biorreatores com sistema fixo, movimento e sob fluxo contínuo. Os biorreatores foram montados utilizando caulinita como matriz de enclausuramento e acetato de sódio, como precursor biossintético, a 0,1; 1,0 e 10mM. Em todos os sistemas observou-se a presença dos compostos fenólicos liquênicos nas três concentrações. O sistema fixo apresentou um pico máximo de produção nas primeiras 24h após a imobilização celular, com acentuada redução ao término de 144h e nenhuma após 1080h (45 dias). O sistema em movimento também obteve um pico máximo de produção nas primeiras 24h com marcante redução em seguida, mantendo-se parcialmente constante durante 360h (15 dias) e decaindo até o término do experimento (2 meses). O sistema sob fluxo contínuo também apresentou produção máxima nas primeiras 24h, com destaque para a concentração de 1,0mM de NaOAc, onde as células mantiveram-se viáveis durante todo experimento. No sistema fixo e em movimento as diferentes concentrações do precursor praticamente não influenciaram a produção dos fenóis, já no sistema sob fluxo contínuo as concentrações de 0,1 e 1,0mM foram as que mais se destacaram. As amostras resultantes foram analisadas por cromatografia em camada delgada (CCD). Sendo possível concluir que os três sistemas se mostraram viáveis para a produção dos metabólitos liquênicos e que a concentração do precursor não influenciou na produtividade das células liquênicas nos sistemas fixo e em movimento. Contudo, o sistema sob fluxo contínuo com 1,0mM de NaOAc mostrou-se mais eficiente que os demais.

*Parte de dissertação de mestrado. Apoio: CNPq

9.1.2 Trabalho enviado a VIII Reunião Regional da SBPQ
Natal, RN – Dezembro, 2006

BIOPRODUCTION OF METABOLITES OF *Canoparmelia texana* (TUCK.) ELIX & HALE FROM CELLULAR IMMOBILIZATION

Silva, N. H.[2]; Pereira, E. C. G.[3] ; Eliasaro, S.[4].; Wessen, C. K[1]
Serafim, A. T. N.[1]
[1]Curso de Mestrado em Bioquímica/UFPE; [2]Departamento de Bioquímica/UFPE; [3]Departamento de Ciências Geográficas/UFPE; [4]Departamento de Botânica/UFPR.

Since centuries ago lichens have been used as perfum, fixative and antibiotics, among other utilities. Due to its application in a large scale, huge amounts of lichen are destroyed, and its reposition is very slow. This way the collect should be very criterious for do not extinguish the lichenized micota. This study had as goal produce metabolites of *Canoparmelia texana* through pieces of stem *in natura* in bioreactores with system in repose (traditional), with movement and under continious flux. Bioreactores were mounted using kaolinite as matrix of inclosure and sodium acetate to 0,1mM, 1,0mM and 10,0mM as precursor biosynthetic. The immobilized liquenics cells in the diverse systems had all kept its vitality for the experiment. The three systems and the three concentrations of the precursor if had shown efficient in the bioproduction, with a discrete prominence for the system in movement and the concentration of 1,0mM, observing it presence of liquenics phenolics composts in the same. In agreement the chromatographic results, gotten from chromatography in thin layer, are bioproduced similar phenolics composts to found in the stem *in natura*, special the atronorin and the divaricatic acid.

Supported by**: CNPq**

Key words: *Canoparmelia texana*, cellular immobilization, lichenic substances

9.2 Normas para a publicação do artigo na Biotechnology Letters

Biotechnology Letters

Description

Biotechnology Letters is the world's leading rapid-publication primary journal dedicated to biotechnology as a whole - that is to topics relating to actual or potential applications of biological reactions affected by microbial, plant or animal cells and biocatalysts derived from them. All relevant aspects of genetics and cell biochemistry, of process and reactor design, of pre- and post-treatment steps, and of manufacturing or service operations are therefore included. Contributions from industrial and academic laboratories are equally welcome. Criteria for the acceptance of papers relate to our aim of publishing useful and informative results that will be of value to other workers in related fields. The emphasis is very much on novelty and immediacy in order to justify rapid publication of authors' results. Short mini-reviews on topics of high current interest are now published in the Journal. These should fit the general format and style of the Journal and normally be no longer than 6 printed pages. Authors intending to submit such a review should contact the Editor beforehand to ensure its suitability for inclusion. Reviews will be peer-reviewed in the normal manner.

Impact factor: 1.108 (2005)

Section "Biotechnology & applied microbiology": Rank 89 of 139

Abstracted/Indexed in:

Agricultural and Environmental Biotechnology Abstracts, ASFA 1, Biological Sciences and Living Resources, ASFA 3, Aquatic Pollution and Environmental Quality, BIOBASE, BioEngineering Abstracts, Biological Abstracts, BIOSIS, Biotechnology Citation Index, CAB Abstracts, CABS, Cambridge Scientific Abstracts, Chemical Abstracts Service, Compendex, Current Contents/ Agriculture, Biology & Environmental Sciences, Current Contents/ Life Sciences, EMBASE, Environmental Engineering Abstracts, Index Medicus/MEDLINE, ISI Alerting Services, Medical and Pharmaceutical Biotechnology Abstracts Databases, Pollution Abstracts, Reference Update, Science Citation Index, Science Citation Index Expanded, SCOPUS

Instructions for Authors

Aims and Scope

Biotechnology Letters is the world’s leading rapid-publication primary journal dedicated to biotechnology as a whole - that is to topics relating to actual or potential applications of biological reactions carried out by microbial, plant or animal cells and biocatalysts derived from them. All relevant aspects of genetics, molecular biology and cell biochemistry, of process and reactor design, of pre- and post-treatment steps, and of manufacturing or service operations are therefore included. Contributions from industrial and academic laboratories are equally welcome. Criteria for the acceptance of papers relate to our aim of publishing useful and informative results that will be of value to other workers in related fields. The emphasis is very much on novelty and immediacy in order to justify rapid publication of authors’ results.

Manuscript Submission

There are no page charges or charges for the publication of colour illustrations for papers published in Biotechnology Letters. It is essential that all co-authors of a paper have seen and have agreed to the submission of the paper. A statement to this effect should accompany the submission. Papers may be submitted electronically (see below) or by post. As Biotechnology Letters is a rapid publication journal, we print directly from authors’ disks. No proofs are

issued to authors. It is therefore imperative that authors ensure their papers are accurate and comply with the following instructions as closely as possible. The Editorial Policy is not to re-write poorly presented papers which will be returned to the authors without assessment. It is a condition of submission of a paper that it is not under consideration whole or in part by another journal nor has it already appeared in print, in whole or in part, in any language, in any other journal.

1. Prepare two error-free manuscripts with two disks, or an e-mail attachment in Word or Wordperfect (not Mac), as well as a covering letter indicating the novelty, urgency or timeliness of your communication. Include copies of any papers referred to as 'in press' or which may have appeared recently and are relevant to the paper under consideration.
2. Mail or e-mail one manuscript to the most appropriate member of the Editorial Board (please see list at journal homepage), who will then make arrangements for refereeing. Inform the Editor or Editorial Secretary to whom the paper has been sent for review.
3. At the same time, mail one copy with the two disks of the manuscript or e-mail the attachment, to the Editorial Secretary:

Mrs E.J. Sparke
Editorial Assistant for Biotechnology Letters
Department of Biological Sciences
University of Hull
Hull HU6 7RX
U.K.

E-mail: E.J.Sparke@hull.ac.uk

Language
The Editor and members of the Editorial Board are unable to undertake extensive rewriting of poorly written papers. If English is not your first language, we would urge you to have your paper thoroughly checked, preferably by a native English speaker, before it is submitted. Papers not reaching a satisfactory standard will be returned to the authors without assessment.

Manuscript Presentation
The maximum printed length of paper in the Journal is normally six pages. There is, however, no limit as to the number of tables or figures that may be used; as long as they will all fit within the limit of six printed pages authors are free to use as many or as few as they consider to be appropriate. The journal's language is English. British English or American English spelling and terminology may be used, but either one should be followed consistently throughout the article. Manuscripts should be printed or typewritten on A4 or US Letter bond paper, one side only, leaving adequate margins on all sides to allow reviewers remarks. Please ensure double-spaced typing of all material, including tables, legends to figures, notes to tables and references. Quotations of more than 40 words should be set off clearly, either by indenting the left-hand margin or by using a smaller typeface. Use double quotation marks for direct quotations and single quotation marks for quotations within quotations and for words or phrases used in a special sense.

Number all the pages (including tables and figures) consecutively with the first page containing:

• title
• author(s)
• affiliation(s)
• the author to whom correspondence is to be addressed should be indicated by an asterisk (*) together with his/her fax number and e-mail address but NOT telephone number, only one author should be listed
• key words
• then leave a space of 4 cm for insertion (by Editor) of handling dates

Title
This must be informative and give key details of the work described. Do not use phrases such as “The effect of...” or “Study of ...”. Please give an explicit clear title and include mention of the biological system being used.

Abstract
Please provide a short abstract that will occupy not more than 5 printed lines in the final printed paper. The abstract should not contain any undefined non-standard abbreviations or unspecified references. Imprecise or qualitative statements should be avoided as should redundant words and phrases. Quantitative values of key data should always be given. Please avoid beginning with a phrase such as “This study shows…”, or “We show in this paper…”.

Symbols and Units
Mathematical units should be set out economically but unambiguously. A solidus (/) can be used for simple fractions or units − for example ml/h − but the double solidus is to be avoided − for example kg/m3h is correct but g/l/h is not. Units can also be given as g l-1 etc. but the style must be consistent. Terms such as ppm are not encouraged; use mg l-1 etc. as appropriate. Concentrations should be given, for example, as g ethanol l-1 not g l-1 ethanol. Uncommon units, symbols and all non-standard abbreviations must be defined at their first appearance but the minimum number of abbreviations is preferred. A list of mathematical terms, if needed, should be provided before the Introduction.

Section Headings
First-, second-, third-, and fourth-order headings should be clearly distinguishable but not numbered.

Appendices
Any supplementary material should be collected in an Appendix and placed before the Reference section.

Notes (footnotes for indicating essential information about the work or origins of the paper

Please use footnotes rather than endnotes. Notes should be indicated by consecutive superscript numbers in the text. A source reference note should be indicated by means of an asterisk after the title. This note should be placed at the bottom of the first typed page.

Introduction
Please keep this as short as possible (usually no more than one typed page); do not give a mini-review of the literature; give key references to recent, relevant publications; historical references are rarely useful. Space is precious - keep it for your results.

Methods
Give information concerning the key protocols only. Avoid describing routine or trivial matters such as how the micro-organisms are maintained, how and when the cultures were sampled. Suppliers of chemicals and manufacturers of equipment should only be given if these are not generally available or are in some way unusual or are crucial for success. Suppliers such as Sigma, Aldrich etc. are given without addresses. There is no need to give references to standard procedures, e.g. Lowry or Bradford methods etc. We encourage authors to place as much relevant information in the footnotes and legends of their tables and figures to increase understanding of these illustrations.

Results
Results given in tables and figures do not need to be described again at length in the text. Focus the reader's attention on your key results. Avoid repetition of the data that is given clearly in the tables and figures. The Results and Discussion sections may be combined. The ideal Results section would simply say: "The results are given in Tables 1 and 2 and further details are shown in Figures 1 and 2."

Discussion
Put your key results into the context of current information, avoid unwarranted or unsupported speculations. Keep your discussion short and focused on explaining the significance of your results.

Tables
Tables should be carefully selected to illustrate key points or data that cannot be easily described in the text. Each table should be numbered consecutively (1, 2, etc.). In tables, footnotes are preferable to long explanatory material in either the heading or body of the table. Such explanatory footnotes, identified by superscript letters, a, b, c etc., should be placed immediately below the table. Please place the title (without abbreviations) at the top on the table itself.

The lay-out of a table should be suitable for printing as either single column (7.5 cm) or double column (16 cm) width. Do not use vertical rulings (lines) and keep horizontal rulings to a minimum. Please ensure 100% relative values are also given in actual, absolute values in a footnote.

Do not quote values beyond the inherent accuracy (or inaccuracy) of the methods. State clearly how many times each complete experiment was done and how many times each individual determination was done. Data given in Tables and Figures should be understandable without recourse to the text: you can add key information regarding experimental procedures in footnotes and omit such details from the Methods section. It is essential that all non-standard abbreviations used in the Table be re-defined.

Indicate the approximate position of the Table by a note in the margin of the text.

Figures
Figures should be carefully selected to illustrate key points or data that cannot be easily stated in the text. For example, it is rarely necessary to give a Lineweaver-Burk plot of an enzyme's activity; its Km and V max values can be easily stated in the text. Similarly with pH and temperature optima for reactions, these can usually be stated within the text.

All photographs, graphs, diagrams and schemes should be referred to as a 'Figure' and they should be numbered consecutively (1, 2, etc.). Multi-part figures should be labelled with upper case letters (A, B, etc.). Scale bars are inserted directly in any photographs or photomicrographs. Relatively small text and great variation in text sizes within figures should be avoided as figures are often reduced in size when printed. Figures will be sized to fit a single column width of the Journal; i.e. 7.5 cm width. Exceptionally, figures may be made to fit a double column width, e.g. 16 cm. Authors must take these sizes into account when designing their figures. All lines on a figure must be of equal thickness and of equal blackness. A detailed legend (without abbreviations) should be provided for each figure. It is preferable to give a full explanation of the protocol being used in the experiment being described in the figure. This information need not then be given in the Methods section. All figures must be referred to in the text; its approximate location in the margin should also be indicated – a handwritten note is acceptable. Please place the legends in the manuscript after the references and tables and on a page separate to the figure(s) themselves.
Original or good quality printouts of all figures should be submitted with the manuscript. Computer-generated printouts should be flawless and on good quality paper. Non-computer generated line figures should be drawn with Indian ink on white matt paper or tracing paper.

Photographs
These must be high-contrast, black and white prints that will show the key details when printed. Colour photographs may be printed without charge. For gels it is often advisable to have a line drawing placed alongside the actual photograph. Do not copy print-outs from recorders or computers attached to instruments - these are rarely of publishable quality. Do not scan-in material as it is impossible for the typesetter to scan-in such material. Avoid making black and white prints from coloured photographs - these do not have sufficient contrast. For photomicrographs, scale bars must be added on the print itself and the magnification given in the legend.

You should also supply the electronic versions of your figures. We prefer figures in TIFF, EPS, PS, PDF and Microsoft Office format. Be aware that we may not be able to use other file formats and that the hard copy may be scanned instead. For vector graphics, EPS is the preferred format. For bitmapped graphics, TIFF is the preferred format. The following resolutions are optimal: line figures - 600 - 1200 dpi; photographs - 300 dpi; screen dumps - leave as is. Colour figures can be submitted in the RGB colour system. Font-related problems can be avoided by using standard fonts such as Times Roman, Courier and Helvetica. Figures should be saved in separate files. The figure legends should be included in the text file.

Acknowledgements
Acknowledgements of people, grants, funds, etc. should be placed in a separate section before the References.

References
References should be given at the end of the typescript arranged alphabetically in order by the last name of the first author and in the form and style indicated below.

1. Journal article:
Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

2. Journal issue with issue editor:
Smith J (ed) (1998) Rodent genes. Mod Genomics J 14:126–233

3. Journal issue with no issue editor:
Mod Genomics J (1998) Rodent genes. Mod Genomics J 14:126–233

4. Book chapter:
Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York

5. Book, authored:
South J, Blass B (2001) The future of modern genomics. Blackwell, London

6. Book, edited:
Smith J, Brown B (eds) (2001) The demise of modern genomics. Blackwell, London

7. Chapter in a book in a series without volume titles:
Schmidt H (1989) Testing results. In: Hutzinger O (ed) Handbook of environmental chemistry, vol 2E. Springer, Berlin Heidelberg New York, p 111

8. Chapter in a book in a series with volume title:
Smith SE (1976) Neuromuscular blocking drugs in man. In: Zaimis E (ed) Neuromuscular junction. Handbook of experimental pharmacology, vol 42. Springer, Berlin Heidelberg New York, pp593–660

9. Proceedings as a book (in a series and subseries):
Zowghi D et al (1996) A framework for reasoning about requirements in evolution. In: Foo N, Goebel R (eds) PRICAI'96: topics in artificial intelligence. 4th Pacific Rim conference on artificial intelligence, Cairns, August 1996. Lecture notes in computer science (Lecture notes in artificial intelligence), vol 1114. Springer, Berlin Heidelberg New York, p 157

10. Proceedings with an editor (without a publisher):
Aaron M (1999) The future of genomics. In: Williams H (ed) Proceedings of the genomic researchers, Boston, 1999

11. Proceedings without an editor (without a publisher):
Chung S-T, Morris RL (1978) Isolation and characterization of plasmid deoxyribonucleic acid from Streptomyces fradiae. In: Abstracts of the 3rd international symposium on the genetics of industrial microorganisms, University of Wisconsin, Madison, 4–9 June 1978

12. Paper presented at a conference:
Chung S-T, Morris RL (1978) Isolation and characterization of plasmid deoxyribonucleic acid from Streptomyces fradiae. Paper presented at the 3rd international symposium on the genetics of industrial microorganisms, University of Wisconsin, Madison, 4–9 June 1978

13. Patent:
Norman LO (1998) Lightning rods. US Patent 4,379,752, 9 Sept 1998

14. Institutional author (book):

International Anatomical Nomenclature Committee (1966) Nomina anatomica. Excerpta Medica, Amsterdam

15. Non-English publication cited in an English publication:
Wolf GH, Lehman P-F (1976) Atlas der Anatomie, vol 4/3, 4th edn. Fischer, Berlin. [NB: Use the language of the primary document, not that of the reference for "vol" etc.!]

16. Non-Latin alphabet publication:
The English translation is optional.
Marikhin VY, Myasnikova LP (1977) Nadmolekulyarnaya struktura polimerov (The supramolecular structure of polymers). Khimiya, Leningrad

17. Published and In press articles with or without DOI:
17.1 In press
Wilson M et al (2006) References. In: Wilson M (ed) Style manual. Springer, Berlin Heidelberg New York (in press)
17.2. Article by DOI (with page numbers)
Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med 78:74–80. DOI 10.1007/s001090000086
17.3. Article by DOI (before issue publication with page numbers)
Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med (in press). DOI 10.1007/s001090000086
17.4. Article in electronic journal by DOI (no paginated version)
Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. Dig J Mol Med. DOI 10.1007/s801090000086

18. Internet publication/Online document

Doe J (1999) Title of subordinate document. In: The dictionary of substances and their effects. Royal Society of Chemistry.Available via DIALOG. http://www.rsc.org/dose/title of subordinate document. Cited 15 Jan 1999

19.1. Online database
Healthwise Knowledgebase (1998) US Pharmacopeia, Rockville. http://www.healthwise.org. Cited 21 Sept 1998
Supplementary material/private homepage
Doe J (2000) Title of supplementary material. http://www.privatehomepage.com. Cited 22 Feb 2000
University site
Doe J (1999) Title of preprint. http://www.uni-heidelberg.de/mydata.html. Cited 25 Dec 1999

FTP site
Doe J (1999) Trivial HTTP, RFC2169. ftp://ftp.isi.edu/in-notes/rfc2169.txt. Cited 12 Nov 1999
Organization site
ISSN International Centre (1999) Global ISSN database. http://www.issn.org. Cited 20 Feb 2000

General Points

Reproducibility of results:

The number of times a complete experiment has been carried out, together with the number of samples analysed on each occasion, should be indicated either in the Methods or in the Tables or Figures. The range of values should be indicated by ± in a table, or by an error-bar in a figure. Standard deviations (S.D.) should be given when appropriate.

Accuracy:

All values should be quoted within the experimental accuracy of the protocol being used, taking into account the type of analysis and instrumentation being used.

Please avoid using 'reduce' when you mean 'decrease' or 'lower' particularly in the context where there may be (bio)chemical reductions.

Units: please always leave a space between the number and the unit; e.g. 37 ˚C not 37˚C.

Avoid 'ppm' and where possible '%' but give as mg/l or g/l etc. SI units and permitted alternatives are to be used. Use correct abbreviations for standard units: h not hr, g not gr etc. If you use % always state if v/v, w/v, v/w or w/w. Abbreviations are never made plural. Do not use normalities for solutions; molarities (M) should be given instead.

Avoid redundant words or phrases such as 'a blue colour', 'at a temperature of 30°C', 'at a wavelength of 340 nm', 'at a concentration of 2 g/l etc.' (Blue is a colour and does not need stating!) Also phrases such as 'It was observed that…' are also redundant and may, without exception, be deleted.

Concentrations are given as 10 g ethanol/l not 10 g/l ethanol; 5 mg kanamycin/ml etc. Mixtures of solvents are given as chloroform/methanol (2:1, v/v) or methanol/water/acetic acid (60:35:5, by vol.).

For presentation of cell growth, please give as dry weight values for both microbes and plant cell cultures. Optical densities (or turbidities) must be converted to the corresponding cell dry wt values. Please do not say "exponential (or logarithmic) growth" unless you have clear data to support that such rates were achieved. Most frequently, arithmetic growth rates are attained in most cell growth systems.

Please use non-standard abbreviations sparingly: these confuse more often than not and are unlikely to save more than 1 or 2 lines of space in the whole paper. Only use if they are replacing lengthy phrases or chemical names.

Checklist

A Checklist for authors is now provided in each issue of the Journal. This should be completed by the submitting author and included with the paper.

Proofs
No proofs are issued to authors. It is therefore imperative that the text of a submitted paper be thoroughly checked before submission. Proofs are, however, checked by the publisher (not the editor).

Offprints
Fifty offprints of each article will be provided free of charge. An order form for additional offprints will be sent to the corresponding author.

Copyright
Authors will be asked, upon acceptance of an article, to transfer copyright of the article to the Publisher. This will ensure the widest possible dissemination of information under copyright laws.

Permissions
It is the responsibility of the author to obtain written permission for a quotation from unpublished material, or for all quotations in excess of 250 words in one extract or 500 words in total from any work still in copyright, and for the reprinting of figures, tables or poems from unpublished or copyrighted material.

Springer Open Choice
In addition to the normal publication process (whereby an article is submitted to the journal and access to that article is granted to customers who have purchased a subscription), Springer now provides an alternative publishing option: Springer Open Choice. A Springer Open Choice article receives all the benefits of a regular subscription-based article, but in addition is made available publicly through Springer's online platform SpringerLink. To publish via Springer Open Choice, upon acceptance please visit www.springer.com/openchoice to complete the relevant order form and provide the required payment information. Payment must be received in full before publication or articles will publish as regular subscription-model articles. We regret that Springer Open Choice cannot be ordered for published articles.

9.3 Normas para a publicação do artigo na Plant Cell Reports

Plant Cell Reports

Plant Cell Reports publishes original, peer-reviewed articles dealing with new advances concerning all aspects of fundamental research and technology in plant cell science and in plant genetics and molecular biology, which includes the following topics:
* genomics and genetics
* cloning
* biochemistry
* physiology
* phytopathology
* morphogenesis
* gene transfer and expression
* abiotic and biotic stress

Plant Cell Reports also publishes review articles on the latest developments and new advances in research and technology in plant molecular biology and biotechnology.
Accepted manuscripts will contribute a significant new advance to a clearly identified technological problem or biological question. It is expected that the findings are relevant beyond the narrow topic of the contribution, and be of interest to a readership with widespread scientific background.
Impact factor: 2.173 (2005)
Section "Plant sciences": Rank 28 of 144
Abstracted/Indexed in:
AGRICOLA, Agricultural and Environmental Biotechnology Abstracts, BIOSIS, CAB Abstracts, CABI Global Health Abstracts, Chemical Abstracts Service, Compendex, CSA Life Sciences Collection, Current Contents/ Agriculture, Biology & Environmental Sciences, Index Medicus/MEDLINE, PaperChem, PASCAL, Science Citation Index, Science Citation Index Expanded, SCOPUS

Instructions for authors

Legal requirements

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, or thesis); that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities - tacitly or explicitly - at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

The "Copyright Transfer Statement" has to be signed and faxed to the publisher together with the corrected proofs (see below) with which it will be provided by the publisher shortly after the manuscript has been accepted for publication.

Manuscript submission

Authors should submit their articles to "Plant Cell Reports" online to facilitate even quicker and more efficient processing. Electronic submission substantially reduces the editorial processing and reviewing time and shortens overall publication time.
The journal publishes short original articles and invited reviews (see next paragraph).

Papers are published in the following sections:

1. Cell Biology and Morphogenesis
2. Genetic Transformation and Cell Hybridization
3. Genetics and Genomics
4. Physiology and Biochemistry
5. Biotic and Abiotic Stress

During submission of your manuscript, you will be asked to state the section in which you would prefer to have your paper published.
To help authors to identify an appropriate editor, numbers corresponding to those in front of the topics listed above appear next to the editors' names.
Manuscripts sent to authors for revision should be returned within 60 days; otherwise they will be treated as new submissions.
Please log directly onto the link below and upload your manuscript (total size of submission 15 Mb maximum) following the instructions given on screen.

Information for Review Authors
"Plant Cell Reports" publishes timely reviews on major developments in all areas of plant cell biology.

Prospective authors may provide a short outline (one or two pages) of the proposed review.

Contact: Prakash P. Kumar, Reviews Editor, E-mail: dbskumar@nus.edu.sg

The proposal will be considered by the Managing and Reviews Editor. The Reviews Editor will inform the authors of the acceptance of the manuscript proposal.

The general instructions for authors should be used for all technical aspects of manuscript preparation. The "Materials and methods" and "Results" sections are not needed, but please give an introduction before proceeding to the details and use informative headings for the different parts of your review.
Reviews should not be longer than seven printed pages, including references, tables, and figures (approx. 21 manuscript pages, or 5,000 words).

Review Authors will not be charged for printing essential color figures.

Manuscript preparation
All manuscripts are subject to peer review and copy editing.

Manuscripts must be written in English and should be typed in double-line spacing throughout with at least 2.5 cm (1 inch) margins.
Use a normal, plain font (e.g., Times New Roman) for text.

Use the automatic page numbering function to number the pages.

For indents use tab stops or other commands, not the space bar.

Use the equation editor of your word processing program or MathType for equations.

Abbreviations should be avoided as much as possible. All abbreviations must be defined in the text where they are used for the first time. The used abbreviations should be listed in a separate list which will be printed after the abstract and the list of key words.

SI units should be used throughout except where non-SI units are more common [e.g. liter (l) for volume].

Please arrange your manuscript as follows:

Title page

Including name(s) of author(s), a concise and informative title, affiliation(s) of the author(s), e-mail address, telephone and fax numbers of the corresponding author.

Abstract

Each paper must be preceded by an abstract presenting the most important results and conclusions in no more than 250 words.

Keywords

Three to five keywords should be supplied after the abstract for indexing purposes.

Introduction

After statement of purpose of the investigation, this section should give a short review of the pertinent literature.

Materials and methods

Following the Introduction this section should provide enough information to permit repetition of the experimental work. Citations to previously published methodology may be used, being careful to describe any changes from the published protocol.

Results

This section should describe the outcome of the study. Data should be presented as concisely as possible, where appropriate in the form of tables or figures. However, very large tables should be avoided.

Discussion

The results should be interpreted with reference to significant work by other authors.

Acknowledgement(s)

References

The list of references should only include peer-reviewed works that are cited in the text and that have been published or accepted for publication. Personal communications should only be mentioned in the text.

Journal titles should be abbreviated in accordance with international standards.

In the text, references should be cited by author and year (e.g. Hammer 1994; Hammer and Sjöqvist 1995; Hammer et al. 1993) and listed in alphabetical order in the reference list.

If available, the Digital Object Identifier (DOI) of the cited literature should be added at the end of the reference in question.

Examples:

* Journal articles:

Kamo K, Jones B, Castillon J, Bolar J, Smith F (2004) Dispersal and size fractionation of embryogenic callus increases the frequency of embryo maturation and conversion in hybrid

tea roses. Plant Cell Rep 22:787-792. DOI 10.1007/s00299-003-0723-5

* Books:
Larcher W (1995) Physiological plant ecology, 3rd edn. Springer, Berlin Heidelberg New York

* Multiauthor books:
Fowke LC, Rennie PJ (1995) Botanical microtechniques for plant cultures. In: Gamborg OL, Phillips GC (eds) Plant cell, tissue and organ culture (fundamental methods). Springer, Berlin Heidelberg New York, pp 217-228
Tables

Figure legends

Tables and figures
Tables must be numbered consecutively with arabic numerals and submitted separately from the text. They should have a title explaining any abbreviation used in that table. Footnotes to tables should be indicated by superscript lower-case letters.
Illustrations must be restricted to the minimum needed to clarify the text. All figures (photographs, graphs or diagrams) should be cited in the text, and numbered consecutively throughout. Figure parts should be identified by lower-case roman letters (a, b, etc.). If illustrations are supplied with uppercase labeling, lower-case letters will still be used in the figure legends and citations.
Figure legends must be brief, self-sufficient explanations of the illustrations. The legends should be placed at the end of the text.
Submit all figures as separate files and do not integrate them within the text. The preferred figure formats are EPS for vector graphics exported from a drawing program and TIFF, or JPG, for halftone illustrations. EPS files must always contain a preview in TIFF of the figure. The file name should include the figure number.

Line drawings
Inscriptions should be legible, with initial capital letters and appropriately scaled to the size of the drawing. Scanned line drawings should be digitized with a resolution of 800 dpi relative to the final figure size.

Halftone illustrations (black and white and color)
Magnification should be indicated by scale bars. For scanned halftone illustrations, a resolution of 300 dpi is usually sufficient.

Color illustrations

Color illustrations will always be published in color in the online version. In print, however, they will only appear in color if the author agrees to make a contribution of Euro 950/US $ 1,150 (plus VAT) per article. Otherwise the figures will be printed in black and white.
Save color illustrations as RGB (8 bits per channel) in TIFF format.

Plates
Several figures or figure parts should be grouped in a plate on one page.

Vector graphics
Fonts used in the vector graphics must be included. Please do not draw with hairlines. The minimum line width is 0.2 mm (i.e., 0.567 pt) relative to the final size.

Size of figures
The figures, including legends, should either match the column width (86 mm) or the print area of 176 x 236 mm. The publisher reserves the right to reduce or enlarge illustrations.

Electronic Supplementary Material
Electronic Supplementary Material (ESM) for a paper will be published in the electronic edition of this journal provided the material is:

submitted in electronic form together with the manuscript

accepted after peer review

ESM may consist of:

information that cannot be printed: animations, video clips, sound recordings (use QuickTime, .avi, .mpeg, animated GIFs, or any other common file format)

information that is more convenient in electronic form: sequences, spectral data, etc.

large quantities of original data that relate to the paper, e.g. additional tables, large numbers of illustrations (color and black & white), etc.

Legends must be brief, self-sufficient explanations of the ESM. ESM is to be numbered and referred to as S1, S2, etc.
After acceptance for publication, ESM will be published as received from the author in the online version only. Reference will be given in the printed version.

Proofreading
Authors are informed by e-mail that a temporary URL has been created from which they can obtain their proofs. Proofreading is the responsibility of the author. Authors should make their proof corrections (formal corrections only) on a printout of the pdf file supplied, checking that the text is complete and that all figures and tables are included. Substantial changes in content, e.g. new results, corrected values, title and authorship are not allowed without the approval of the responsible editor. In such a case please contact the Editorial Office before returning the proofs to the publisher. After online publication, corrections can only be made in exceptional cases and in the form of an Erratum, which will be hyperlinked to the article.

Online First
Papers will be published online about one week after receipt of the corrected proofs. Papers published online can already be cited by their DOI. After release of the printed version, the paper can also be cited by issue and page numbers.

Offprints
Twenty-five offprints of each contribution are supplied free of charge. If you wish to order additional offprints you must return the order form which is provided with the proofs and return it together with the corrected proofs.

Springer Open Choice
In addition to the traditional publication process, Springer now provides an alternative publishing option: Springer Open Choice (Springer's open access model). A Springer Open Choice article receives all the benefits of a regular article, but in addition is made freely available through Springer's online platform SpringerLink. To publish via Springer Open Choice upon acceptance of your manuscript, please click on the link below to complete the relevant order form and provide the required payment information. Payment must be received in full before free access publication.

Copyright information

Submission of a manuscript implies: that the work described has not been published before (except in form of an abstract or as part of a published lecture, review or thesis); that it is not under consideration for publication elsewhere; that its publication has been approved by all co-authors, if any, as well as - tacitly or explicitly - by the responsible authorities at the institution where the work was carried out. The author warrants that his/her contribution is original and that he/she has full power to make this grant. The author signs for and accepts