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FABRÍCIO HOLANDA E HOLANDA

Biodegradação do cloranfenicol por fungos endofíticos isolados de *Bertholletia excelsa* (Castanha-do-Brasil)

Macapá
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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal do Amapá para obtenção do Título de Mestre em Ciências Farmacêuticas.

Orientador: Prof. Dr. Irlon Maciel Ferreira
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Programa de Pós-Graduação em Ciências Farmacêuticasda Universidade Federal do Amapá

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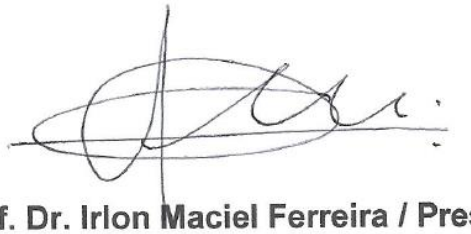

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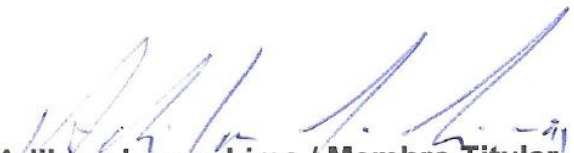

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Dedico este trabalho às mulheres da minha vida, Juventina Viana Holanda e Nayana Duarte da Silva Holanda.

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SUMÁRIO

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SÍMBOLOS, SIGLAS E ABREVIATURAS

ANVISA	Agência Nacional de Vigilância Sanitária
BD	Biodegradação
B.O.D	Demanda Bioquímica de Oxigênio
CAP	Cloranfenicol
CEF	Cefitiofur
CG	Cromatografia gasosa
CMI	Concentração Mínima Inibitória
DBPs	Subproduto de desinfecção
DMSO	Dimetilsulfóxido
ERN	Enrofloxacina
HMNsFP	Halonitrometanos
HPAs	Hidrocarbonetos aromáticos policíclos
HPLC	Cromatografia Líquida de Alta Eficiência.
MS	Espectrômetro de Massa.
Rpm	Rotação por minuto.
SMX	Sulfametoxazol
SMZ	Sulfametoxazol
UV	Ultravioleta.

RESUMO

Biodegradação do cloranfenicol por fungos endofíticos isolados de *Bertholletia excelsa* (Castanha-do-Brasil)

Introdução: Antibióticos é um grupo de micropoluentes de grande risco para os ecossistemas e contribuem para o desenvolvimento de resistência em cepas bacterianas, quando descartada de forma incorreta ou pelo uso indiscriminado. O cloranfenicol, composto abordado neste estudo, resiste aos procedimentos convencionais de degradação no tratamento de água residual. Assim, o processo de biodegradação empregando microrganismos específicos e eficientes, incluindo fungos filamentosos, é uma opção ecologicamente viável e de baixo custo, que já vem sendo empregado com sucesso para biodegradação de outros agentes químicos. **Objetivo:** Portanto, o objetivo deste estudo foi avaliar a biodegradabilidade da molécula decloranfenicol por cinco linhagens de fungos endofíticos isolados de *Bertholletia excelsa* coletados na Amazônia brasileira. **Metodologia:** Para isso, as cepas dos fungos BIORG 4, BIORG 5, BIORG 6, BIORG 7 e BIORG 9 foram triadas em meio sólido / líquido e o delineamento experimental foi realizado para otimizar as condições de cultivo, variando-se o pH do meio, a concentração de cloranfenicol e o tempo de reação. Análises por Cromatográfia Líquida de Alta Eficiência (HPLC-UV) foi realizada para quantificação do teor de biodegradação e a Cromatografia Gasosa acoplada a Espectrometria de Massas (GC-MS) foi empregada para detecção e identificação de metabólitos. Além disso, uma avaliação toxicológica ambiental foi realizada utilizando a alga *Chlorella vulgaris*. **Resultados e Discussões:** Os resultados das culturas desses fungos em meio sólido demonstraram que o cloranfenicol afetou consideravelmente o crescimento das cepas. Além disso, a escarificação inicial da biodegradação em 3, 6 e 9 dias mostrou que todas as cepas conseguiram aumentar a degradação desse antibiótico; *Trichoderma* sp. (BIORG 7), foi a linhagem que apresentou melhores resultados, foi submetida a delineamento experimental (Box-behnken) composto por 15 experimentos, tendo como variáveis: pH (5, 7 e 9), período (24, 48 e 72 horas) e concentração de cloranfenicol (50, 100 e 150 mg.L-1), atingindo um percentual de biodegradação de cerca de 30%. O metabólito 4-nitrobenzaldeído foi identificado e causador da toxicidade a esses microrganismos, um metabólito que pode estar relacionado também com as doenças causadas em diferentes organismos. **Conclusões:** Os fungos endofíticos conseguiram acelerar a biodegradação do cloranfenicol e podem ser melhor estudados em instalações de tratamento de resíduos. Além disso, foi a primeira pesquisa de biodegradação com fungos endofíticos isolados da Amazônia.

Palavras-Chave: Micropoluentes; microrganismos endíticos; biodegradação de antibiótico; 4-nitrobenzaldeído.

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ABSTRACT

Biodegradation of chloramphenicol by endophytic fungi isolated from *Bertholletia excels* (Brazil nuts)

Introduction: Antibiotics are a group of micropollutants at great risk to ecosystems and contribute to the development of resistance in bacterial strains when discarded incorrectly or by indiscriminate use Chloramphenicol, the compound addressed in this study, resists conventional degradation procedures in the treatment of residual water. Thus, the biodegradation process employing specific and efficient microrganisms, including filamentous fungi, is an ecologically viable and low cost option that has already been used successfully for the biodegradation of other chemical agents. **Objective:** The objective of this study was to evaluate the biodegradability of the decoranphenicol molecule by five endophytic fungi isolated from *Bertholletia excelsa* collected in the Brazilian Amazon. **Methodology:** For this, the strains of BIORG 4, BIORG 5, BIORG 6, BIORG 7 and BIORG 9 were screened in solid / liquid medium and the experimental design was performed to optimize the culture conditions by varying the pH of the medium, the chloramphenicol concentration and the reaction time. Analysis by High Performance Liquid Chromatography (HPLC-UV) was performed to quantify the biodegradation content and Gas Chromatography coupled to Mass Spectrometry (GC-MS) was used for the detection and identification of metabolites. In addition, an environmental toxicological assessment was performed using the *Chlorella vulgaris* algae. **Results and Discussion:** Results of fungal cultures on solid medium showed that chloramphenicol significantly affected the growth of fungal strains. In addition, the initial scarification of biodegradation at 3, 6 and 9 days showed that all strains succeeded in increasing the degradation of this antibiotic; *Trichoderma* sp. (5, 7 and 9), period (24, 48 and 72 hours), which was submitted to an experimental design (Box-behnken)) and chloramphenicol concentration (50, 100 and 150 mg.L-1), reaching a biodegradation percentage of about 30%. The metabolite 4-nitrobenzaldehyde was identified and showed toxicity to these microorganisms, a metabolite that may be related to the diseases caused in different organisms. **Conclusions:** Endophytic fungi have been able to accelerate the biodegradation of chloramphenicol and can be better studied in waste treatment facilities. In addition, it was the first biodegradation study with isolated fungi of the Amazon

Keywords: Micropollutants; Plant-microorganism; Antibiotic Biodegradation; 4-nitrobenzaldehyde.

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1 INTRODUÇÃO

1.1. ANTIMICROBIANOS

1.1.1. Aspectos gerais

Em 1928, Alexander Fleming, ao sair de férias do hospital de Londres, esqueceu uma de suas placas de Petri fora da estufa, e notou que uma substância produzida pelo fungo *Penicillium notatum* havia inibido o crescimento da bactéria *Staphylococcus aureus* na placa. Essa substância descoberta ao acaso ficou conhecida como penicilina. No entanto, apesar da penicilina não apresentar ação tóxica sobre o organismo, Fleming não conseguiu produzir esse componente em quantidade suficiente para emprega-lo sistematicamente. (SANTOS, 2007).

A penicilina só teve sua produção em larga escala na década de 1940, após intensivos estudos. Na mesma época foram desenvolvidos a classe dos anfenicóis e dos aminoglicosídeos, e décadas seguintes de 50 e 60 foi a vez das tetraciclinas, macrolideos, glicopeptídeos, rifampicinas, quinolonas e o trimetroprim (TAVARES, 2001). A Tabela 1 detalha as principais descobertas dos antimicrobianos até o ano 2000.

Tabela 1. Cronologia das principais descobertas de antimicrobianos até início do século XXI.

Década	Evento
1920	Descoberta da penicilina.
1930	Descoberta da sulfonamida e gramicidina.
1940	Introdução da penicilina e descoberta da estreptomicina, bacitracina, cefalosporinas, cloranfenicol, clortetraciclina e neomicina.
1950	Descoberta da oxitetraciclina, eritromicina, plomixina, vancomicona e kanamicina.
1960	Descoberta da espectinomicina, gentamicina, clindamicina e fosfomicina. Introdução da meticilina, ampicilina, cefalosporinas, vancomicina e doxicilina.
1970	Descoberta da tobramicina e cefamicinas. Introdução da rifampicina, minociclina, cotrimazol e amicacina.
1980	Descoberta da daptomicina. Introdução da amoxicilina/clavulanato, imipenem/cilastatina e ciprofloxacina.
1990	Relato da linezolida, ketolídios (telitromicina) e glicilciclinas (tigeciclina). Introdução da azitromicina, claritromicina e quinupristina/dalfopristina.
2000	Introdução da linezolida, daptomicina, telitromicina e togeciclina.

Fonte: França (2012)

Os antimicrobianos são substâncias que têm a capacidade de inibir o crescimento e/ou destruir microrganismos. Em geral, são matabólitos secundários produzidos por bactérias ou por fungos, podendo ser total ou parcialmente sintéticos.

Um antimicrobiano é utilizado terapeuticamente para prevenir ou tratar uma infecção, diminuindo ou eliminando os organismos patogênicos e, se possível, preservando os germes da microbiota natural. Para isso é necessário conhecer o tipo de infecção a ser tratada (MELO et al. 2012).

Os antimicrobianos podem ser classificados através de diversas variáveis, conforme a Tabela 2.

Tabela 2. Demonstrativo das variáveis da classificação dos antimicrobianos.

VARIÁVEL	CLASSIFICAÇÃO	EXEMPLO
Microrganismos suscetíveis	Antibacterianos/	Beta-lactâmico
	Antifúngicos	Griseofulvina
	Antivirais	Aciclovir
	Antiparasitários	Pirimetamina
Origem do antimicrobiano	Antibióticos: produzidos por microrganismos	Aminoglicosídeo
	Quimioterápicos: sintetizados em laboratório	Sulfonamidas
Atividade antibacteriana	Bactericida: matam o microrganismo	Quinolona
	Bacteriostático: inibem o crescimento do microrganismo	Macrolídeo
Mecanismo de ação	Alteração da parede celular	Beta-lactâmico
	Alteração da membrana citoplasmática	Anfotericina B
	Interferência na replicação cromossômica	Antifúngicos/antivirais
	Inibição da síntese protéica	Aminoglicosídeo
	Inibição metabólica	Sulfonamidas
Espectro de ação	Espectro para Gram-positivas	Penicilina
	Espectro para Gram-negativas	Aminoglicosídeo
	Amplo espectro	Cloranfenicol
	Ativo sobre protozoários	Tetraciclina
	Ativo sobre fungos	Nistatina
	Ativo sobre espiroquetas	Eritromicina
	Ativo sobre riquétsias, micoplasma e clamídias	Microlídeo
	Ativo sobre micobactérias	Estreptomicina
	Ativo sobre algas	Anfotericina B

Fonte: Melo et al. (2012).

Ao mesmo tempo que os antimicrobianos representam uma importante classe de medicamentos, o uso inapropriado desses fármacos tem acarretado sérias consequências aos pacientes, como: processos alérgicos, dificuldades no manejo de infecções.

Assim, há a necessidade de se reavaliar as práticas hoje exercidas na prescrição dos antimicrobianos e também na dosagem ministrada aos pacientes, a fim de que se estabeleça uma reflexão sobre o uso racional desses medicamentos (FRANÇA, 2012).

O uso indiscriminado de antibióticos, desde a década de 1940, é um dos fatores que acelerou o processo de adaptação dos microrganismos. De acordo com dados da consultoria internacional IMS Health, os antibióticos são a quinta classe de remédio mais vendido do mundo, atrás apenas de drogas de combate ao câncer, dores, diabetes e hipertensão (LOIOLA, 2014).

Nos países em desenvolvimento como é o caso do Brasil, o uso indiscriminado dos antibióticos é mais grave, principalmente devido acesso facilitado na compra sem o uso de receitas, mesmo com a retenção de receitas sendo obrigatória desde novembro de 2010 pela ANVISA.

Porém o uso excessivo dessa classe de medicamento além de contribuir para o desenvolvimento de resistência bacteriana também é responsável pelo considerável aumento dos custos financeiros hospitalares e dos riscos de reações adversas e interações medicamentosas. (RODRIGUES; BERTOLDI, 2010).

1.1.2. Impactos dos antibióticos no ambiente e nos seres vivos

O monitoramento no meio ambiente dos microcontaminantes vem recebendo grande importância da comunidade científica desde o fim da década de 1970 (HIGNITE; AZARNOFF, 1977), especialmente devido ao reconhecimento dos seus efeitos, tais como: toxicidade aquática, genotoxicidade, perturbação endócrina em animais selvagens, seleção de bactérias patogênicas resistentes, bioacumulação, entre outros (HALLING-SØRENSEN et al*.* 1998).

Segundo Suárez et al. (2008), o conhecimento das características dos microcontaminantes é muito importante para o esclarecimento dos mecanismos de degradação e transporte que ocorrem durante o tratamento de esgoto. Além disso, as condições operacionais e configurações das diversas unidades do tratamento podem influenciar nos mecanismos de sorção, fotodegradação, volatilização e transformações químicas e/ou biológicas dos compostos.

De acordo com Santos et al. (2010), os fármacos mais frequentemente detectados em ambientes aquáticos são classificados como anti-inflamatórios não esteroides (16%), antibióticos (15%), reguladores lipídicos (12%) e hormônios sintéticos (9%), que somados perfazem 52% dos 134 artigos publicados entre 1997 e 2009 sobre a ocorrência de fármacos em ambientes aquáticos.

Os antibióticos têm características químicas diversas, e são escassas as informações sobre seus efeitos no ambiente. Porém, o que já se conhece a respeito, pode-se concluir um alto grau de interação com o ambiente.

Os antibióticos são substâncias polares, não voláteis, com estruturas químicas e reações de alta complexidade. Possuem, em suas estruturas químicas, um grande número de grupos funcionais, como o ácido carboxílico, amida, amina, álcool, cetona, enol, fenol, tiazol, nitro composto, derivados halogenados, sulfonamida entre outros, caracterizando uma grande atividade química e biológica dessas substâncias. A presença de grupos funcionais diversificados propicia interações por processos de adsorção ou complexação, dependendo das condições do meio. Por isso podem permear o solo sendo carreados para as águas subterrâneas, e com isso atingir os rios, expondo tanto os organismos aquáticos como os usuários dessa água a esse tipo de contaminação (CASTIGLIONI, et al. 2004).

As indústrias farmacêuticas geram efluentes com concentrações residuais dos produtos sintetizados e/ou envazados, juntamente com os solventes utilizados nos processos e seus produtos intermediários, os quais podem apresentar características de não biodegradabilidade, e toxicidade para o meio ambiente (MASCOLO et al. 2010).

De acordo com Krause (2009) os efeitos causados por esses micropoluentes no meio ambiente são em função de: concentração no ambiente, lipofilicidade, persistência, bioacumulação, tempo de exposição, mecanismos de biotransformação e de excreção. Ocorrem biotransformações de algumas substâncias presentes no meio ambiente, formando subprodutos igualmente ou até mais danosos que as substâncias originais.

O lançamento de efluente contendo antibióticos, em geral, pode levar ao desenvolvimento de bactérias patogênicas resistentes, alterando a estrutura da comunidade microbiana na natureza, e afetando as bactérias suscetíveis. A maior rota de exposição, tanto para os humanos como para animais, é ingerindo fármacos

através dos alimentos ou água, o que pode levar a bioacumulação e biomagnificação, especialmente em direção às espécies no topo da cadeia alimentar.

Os fármacos têm sido detectados em águas potáveis, caracterizando um risco direto para os seres humanos e outros seres vivos, levando ao questionamento da constante contaminação das fontes de água, associado ao processo contínuo e crescente de reutilização da água em todo o mundo. Um grande problema encontrado é a taxa de reposição contínua desses compostos, os quais, potencialmente, sustentam a exposição crônica nos organismos aquáticos (KASPRZYK-HORDERN, 2010).

A pesquisa de Liu e colaboradores (2017) avaliaram 40 antibióticos e seus riscos para a saúde humana, concluindo que os dados obtidos foram úteis para a melhor compreensão dos efeitos a longo prazo na saúde humana. Identificou alto risco para a exposição de antibióticos, principalmente devido as concentrações máximas elevadas e uso combinado dessas substâncias. Contudo, são necessários mais estudos para esclarecimento de tais relações.

1.1.3. Cloranfenicol

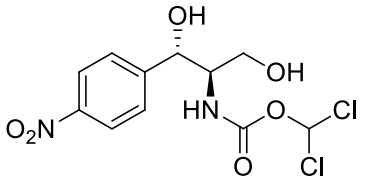
A historia dos anfenicóis começou em 1947 com a identificação do cloranfenicol a partir de *Streptomyces venezuelae*, que se transforma no primeiro antibiótico produzido em grande escala. Embora dotado de um amplo espectro, propriedades farmacocinéticas e formas de dosagem interessante múltiplos, este antibiótico têm experimentado um desenvolvimento limitado por causa da toxicidade hematológica. (EPAULARD; BRION, 2010).

A substância cloranfenicol (CAP), D-(-)-treo-2,2-dicloro-*N*-[β-hidroxi-*α*-(hidroximetil)-*p*-nitrofenil], de massa molar 323,1325 g/mol e fórmula molecular $C_{11}H_{12}Cl_2N_2O_5$ é um antibiótico com classificação bacteriostática de amplo espectro, que foi comumente aplicado como medicamento de uso veterinário e humano devido a suas propriedades de combater uma variedade de microrganismos aeróbios e anaeróbicos. O CAP é uma substância lipossolúvel que se difunde através da membrana celular e se liga de forma reversível à subunidade protéica 50S dos

ribossomos das células de procariontes, inibindo assim a síntese da proteína (JÚNIOR et al. 2006).

A estrutura molecular de cloranfenicol não é particularmente complicado em relação à matriz de moléculas orgânicas utilizadas para fins semelhantes.

Figura 1. Estrutura química do cloranfenicol.



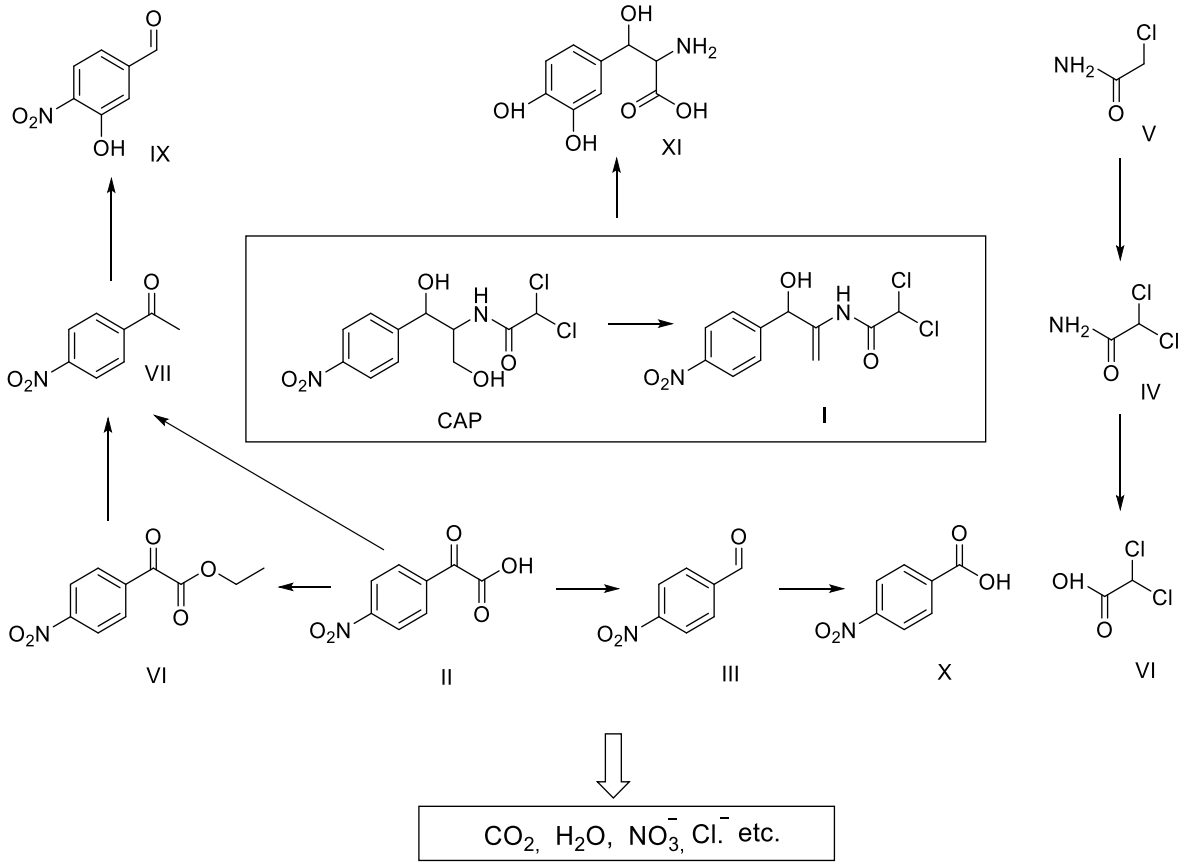
Cloranfenicol

Fonte (próprio autor).

Segundo a ANVISA (2007) o cloranfenicol tem indicação clínica no tratamento de infecções por enterococos resistentes à vancomicina, salmoneloses como a febre tifóide, meningite bacteriana e epiglotite, artrite séptica e osteomielite por *Haemophilus influenzae* em pacientes alérgicos aos ß-lactâmicos e ricketsioses ou erlickiose com ação bacteriostática. É disponível na forma oral, intravenosa e tópica. É hidrolisado no trato digestivo antes de ser absorvido, atingindo pico sérico em 1 a 2 horas. Penetra na maioria dos fluidos orgânicos, incluindo os líquidos pleural, peritoneal e sinovial. Atinge no liquor a metade da concentração plasmática na presença ou não de inflamação das meninges. Por ser lipofílico, alcança no parênquima cerebral concentração até 9 vezes maior que a do plasma.

Em amostras residuais contendo cloranfenicol em sua formualação farmacêutica foi detectado o 4-nitrobenzaldeído por HPLC-UV, conforme Luo et al. (2018). O 4-nitrobenzaldeído é um produto da fotodegradação do cloranfenicol possuindo grande potencial genotóxico e mutagênico em humanos já relatados. Nie et al. (2014) avaliou a degradação do cloranfenicol utilizando persulfato termicamente ativado (TAP), sugerindo uma rota de reação.

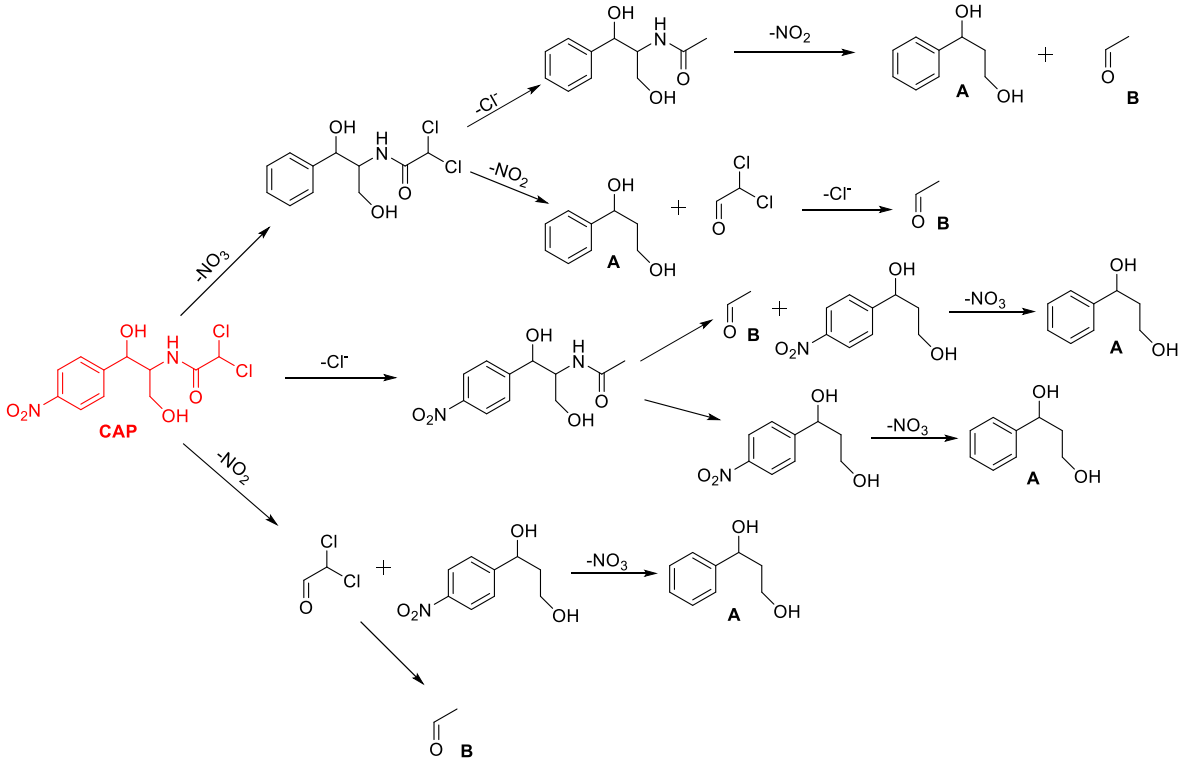
Esquema 1. Provável caminho de reação do cloranfenicol.



Fonte: Adaptado de Nie et al. (2014).

Outro estudo de degradação do cloranfenicol foi realizado por Prado et al. (2010), através de um reator eletroquímico de fluxo, baseado em reação de oxidação. Esse processo levou a formação de produtos como íons cloreto, íons nitrato e nitrito, inferindo uma rota com subprodutos orgânicos e inorgânicos, conforme Esquema 2.

Esquema 2. Rota de formação de possíveis subprodutos derivados da formação de íons inorgânicos.

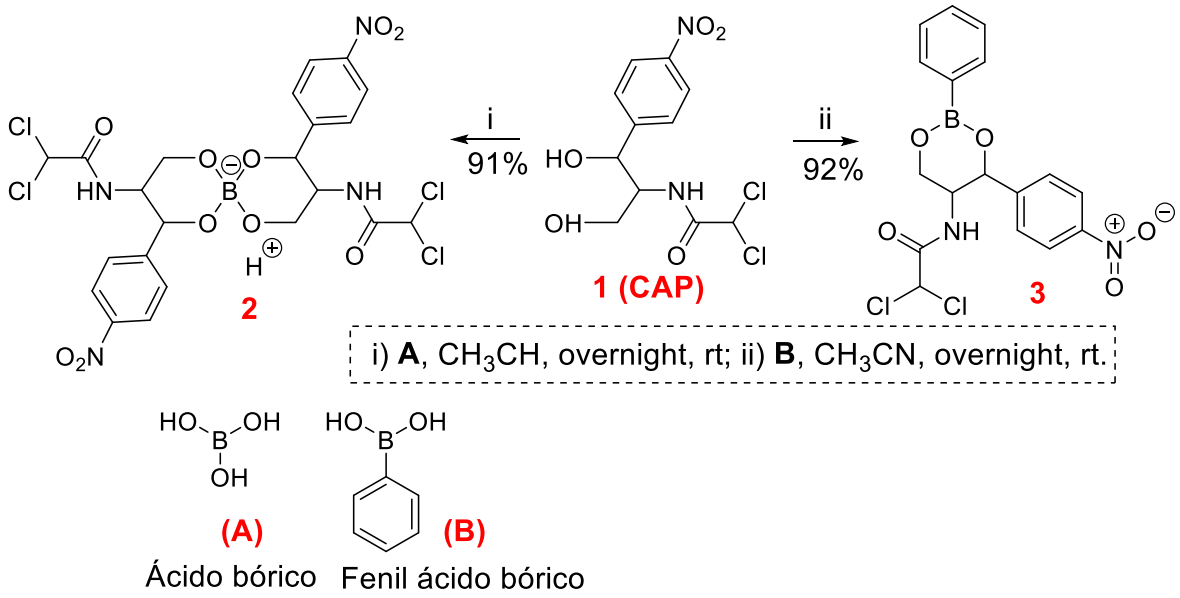


Fonte: Adaptado de Prado et al. (2010).

Outra técnica de degradação do clorandenicol foi estudada por Dong et al. (2017), através de Ultravioleta (UV) / cloro que é considerado um processo de oxidação avançada de micropoluentes emergentes, se mostrando mais eficiente do que apenas aplicando UV para CAP. Além da degradação do cloranfenicol ter sido reforçada por essa técnica, também foi investigado a formação de subprodutos de desinfecção (DBPs) e halonitrometanos (HMNsFP).

Mesmo devido a perca da eficácia em tratamentos terapêuticos por resistância bacteriana, o cloranfenicol vem sendo alvo de pesquisas para biossíntese de novos produtos farmacêuticos, como é caso do estudo de Bhattacharya et al. (2018), com a síntese do borato (2) e fenilboronato (3) derivados de cloranfenicol (1) que apresentaram baixos valores de CMI do que as cepas resistentes ao cloranfenicol.

Esquema 3. Síntese de cloranfenicol borato e fenilboronato .



Fonte: Adaptado de Bhattacharya et al. (2018).

1.2. FUNGOS FILAMENTOSOS

1.2.1. Características gerais dos fungos filamentosos

Para Sotão et al. (2004) os fungos constituem um grupo de organismos com grandes variações morfológicas, com espécies unicelulares e multicelulares, macroscópicas e microscópicas. Alguns são popularmente conhecidos como mofo, bolor, urupê, orelha de pau, leveduras, cogumelo e estrela da terra. Entre os seres vivos são classificados no reino dos fungos (Fungi) e a ciência que os estuda é chamada de Micologia, cujo termo deriva do grego *mykes* = fungo. Estima-se que exista pelo menos um milhão e quinhentas mil espécies de fungos, das quais aproximadamente setenta mil espécies já foram descritas (ESPOSITO; AZEVEDO, 2004).

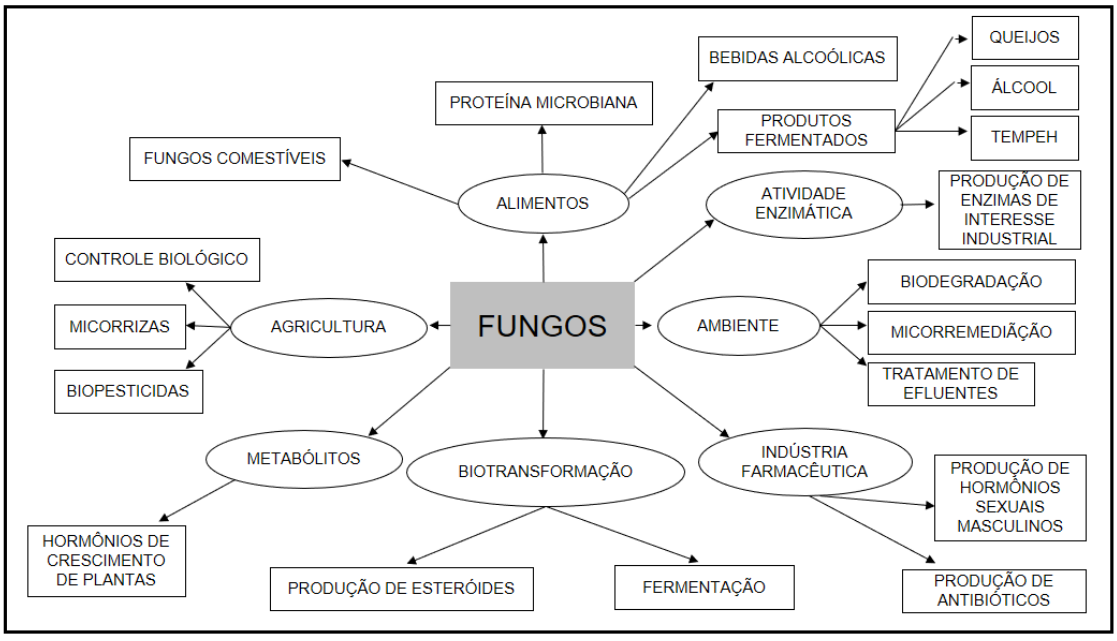
Os fungos apresentam células eucarióticas, com um núcleo distinto e as organelas citoplasmáticas são envolvidas por membranas, o que permite diferenciá-los das bactérias. São heterotróficos, podendo ser saprofíticos ou parasitários. Na sua maioria produzem grandes filamentos chamados de hifas, formando uma massa em seu conjunto, o micélio (BENEVIDES; MARINHO, 2015).

Exibem reprodução sexuada e/ou assexuada de diversas formas, bem como o fenômeno de parassexualidade, que consiste na recombinação genética. As estruturas de reprodução são diferentes das somáticas, exibindo uma variedade de formas, as quais são utilizadas na classificação dos fungos (SILVA, 2006).

Os fungos juntamente com as bactérias heterotróficas são os agentes mais importantes de degradação de matéria orgânica na Terra (ESPOSITO; AZEVEDO, 2004), tendo uma atuação tão importante para a vida no planeta quanto os produtores (RAVEN et al. 2007).

Os fungos são considerados biodegradadores eficientes de polímeros de plantas naturais como lignina e celulose. Contudo, também degradam outros tipos de moléculas orgânicas como ceras, borrachas, fenois, benzeno, tolueno, xileno e xenobióticos presentes em ecossistemas florestais, onde eles são os principais decompositores de substância orgânica. Constituem-se no grupo mais expressivo numericamente, excetuando-se os insetos, distribuídos nos mais diversos habitats. Pode ser encontrado compondo a biota do solo, livres ou associados a vegetais ou outros grupos, atuando principalmente na reciclagem de nutrientes (MODA et al. 2005). A figura a seguir, demonstra as interações, potencial e aplicações dos fungos com as mais diferentes áreas, devido suas características biológicas são utilizados no desenvolvimento de biotecnologias.

Figura 2. Potencial de interação e utilização dos fungos.



Fonte: adaptado de Esposito; Azevedo (2004).

Esses organismos secretam enzimas no substrato onde absorvem as moléculas resultantes da ação dessas enzimas. Com isso, conseguem obter nutrientes para seu crescimento, além de disponibilizarem os produtos resultantes da degradação para ação de outros organismos, e por essa razão são os degradadores primários de material orgânico mais importantes da natureza, participando ativamente nos ciclos de carbono, nitrogênio e fósforo e outros (SILVA, 2006).

1.2.2. Fungos endofíticos

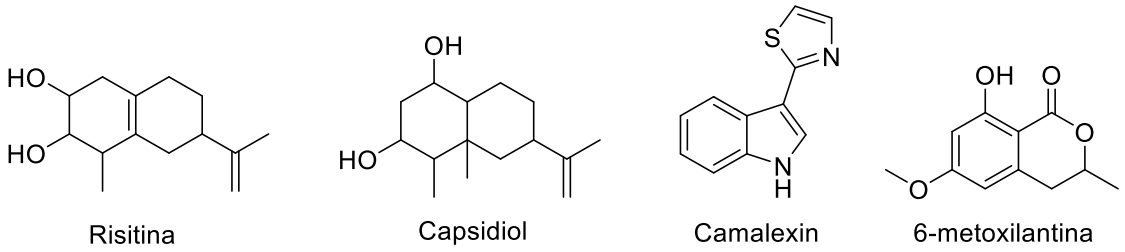
Microrganismos endofíticos são, principalmente fungos e bactérias, que vivem no tecido interior de plantas, habitando de modo geral, suas partes aéreas, como folhas e caules, sem causar aparentemente nenhum dano a seu hospedeiro. Foram mencionados pela primeira vez no início do século XIX. Devido uma série de motivos, começaram a chamar atenção de pesquisadores. Verificou-se que possuíam propriedades de interesse como proteção contra insetos-pragas, outros microrganismos patogênicos e inclusive contra herbívoros. Hoje, sabe-se que endófitos podem produzir toxinas, antibióticos e outros fármacos, e muitos produtos de potencial interesse biotecnológico (AZAVEDO, 1998).

Um dos conceitos mais atuais e abrangentes sobre microrganismos edofíticos é descrito da seguinte maneria: “microrganimos endofíticos são aqueles que podem ou não crescer em meio de cultura, habitando o interior de tecidos vegetais sem causar prejuízo ao hospedeiro e sem produzir estruturas externas emergindo dos vegetais” (ARAÚJO et al. 2002).

Das quase 300 mil espécies de plantas que existem, cada uma destas pode hospedar um ou mais microrganismo endofítico. Isto faz com que este grupo de ser vivo seja explorado por indústrias médicas e farmacêuticas, visto que metabólitos secundários produzidos por eles têm demonstrado potencial (STROBEL et al. 2003).

Exemplo de metabólitos que podem ser induzidos pelos endófitos são as fitoalexinas, substâncias de baixo peso molecular com atividades antimicrobianas, produzido pelas plantas (CHIARAVALLOTI, 1992). Da parte dos fungos pode-se citar a produção de micotoxinas, metabólitos secundários que podem causar doenças em humanos e outros animais (CLAY, 1988).

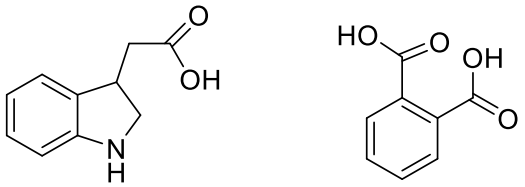
Figura 3. Estrutura química de fitoalexinas.



Fonte: Próprio autor.

Para Sudha (2016) os fungos endofíticos têm ampla aplicação em diferentes campos. Possui o potencial de produzir muitos compostos bioativos, tais como o taxol (anticancerígeno), ácido dodecanóico e ftálico (atividade inseticida), auxina e etileno (importantes para o crescimento das plantas), entre outras. Os metabolitos secundários produzidos pelos fungos endofíticos têm a capacidade de atuar como agente de controle biológico. No futuro, os produtos dos fungos endofíticos serão uma fonte barata para a indústria médica, a agricultura e outras indústrias. É certo que a pesquisa sobre fungos endofíticos levará a isolar compostos mais novos.

Figura 4. Exemplo de substâncias bioativas produzidas por fungos endofíticos.



Fonte: Próprio autor.

Fungos de origem endofítica já foram descritos em diversos estudos de biodegradação. Recentemente Fu et al. (2018) realizaram a biodegradação do fenantreno com o fungo endofítico *Phomopsis liquidanbari*, o fenantreno é considerado um contaminante difuso de hidrocarbonetos aromáticos policíclos (HPAs) com características de carcinogenicidade, teratogenicidade e mutagenicidade. O isolado em questão foi capaz de utilizar esse contaminante como fonte de carbono para crescimento, além de removê-lo *in vitro* de 77,4% em 10 dias.

Nevada; Sanjeev; Kulal (2018) estudaram a biodegradação e desintoxicação do corante recalcitrante de antraquinona por fungo endofítico (*Phomopsis* sp.)

irradiado com feixe de elétrons, o carante foi reduzido para 60% com proposta de rota de biodegradação.

Nos últimos anos outros trabalhos importantes na área da biodegradação e biorremediação foram feitos com fungos endofíticos, alguns já descritos anteriormente como a biodedradação de pesticidas (BIROLLI, 2013), outros como a biodegradação de polietileno e polipropileno (SHEIK, 2015), o poluente ácido ferúlico (XIE; DAI, 2015), além de ensaios com fármacos.

1.3. BIOTRANSFORMAÇÃO

De acordo com Hurst et al. (2007), a biotransformação consiste na alteração da estrutura química de uma substância, tornando-a quimicamente mais simples ou mais complexa, podendo aumentar ou reduzir sua toxicidade, mobilidade e/ou recalcitrância no meio.

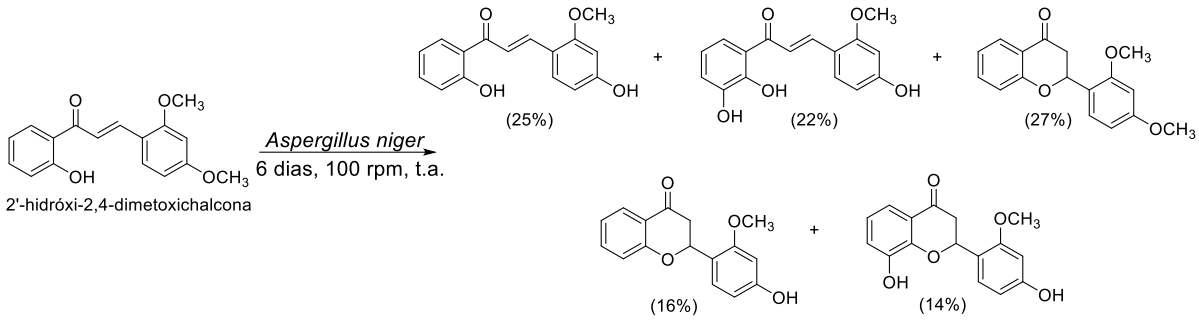
O termo biodegradação refere-se à degradação microbiológica direta ou indireta de um composto orgânico, sendo considerada a principal via de deterioração desses produtos no solo (PRATA, 2002). Os microrganismos utilizam esse composto como substrato pela ação de suas enzimas ou co-enzimas e podem ainda utiliza-los como nutrientes e energia para a sobrevivência (MAIER, 2015). A completa biodegradação ou mineralização envolve a oxidação dos compostos intermediários, que podem ser mais ou menos tóxicos que o inicial.

Os processos de biodegradação de compostos orgânicos ocorrem naturalmente pela ação de bactérias e fungos, que transformam substâncias diversas em outras de menor toxicidade na maioria dos casos, o que despertou o interesse em aplica-lo à ensaios biológicos, e aos poucos vem se tornando uma potencial biotecnologia para ser utilizada por exemplo nos segmentos ambientais, industriais, agrícolas e farmacêutico.

A biotransformação é considerada uma tecnologia econômica e ecologicamente viável e já foi usada para modificar as estruturas de alguns produtos biologicamente ativos e estudar o metabolismo de produtos naturais (CARVALHO, 2006). Muitos dos medicamentos e esteróides com potencialidades biológicas foram sintetizados por transformação microbiana (CHOUDHARY et al. 2007). Os microrganismos também são utilizados para converter os produtos naturais bioativos

em derivados com atividades aprimoradas, como a aplicação da biotransformação para modificar as estruturas de flavonoides naturais com potencial atividade farmacológica, (CAO et al. 2015), como descrito por Alarcón et al. (2013) utilizando o fungo terrestre *Aspergillus niger* para promover a ciclização e hidroxilação de 2'-hidroxichalconas metoxiladas, de acordo com o Esquema 4.

Esquema 4. Biotransformação de 2'-hidroxichalconas metoxiladas pelo fungo *Aspergillus niger.*

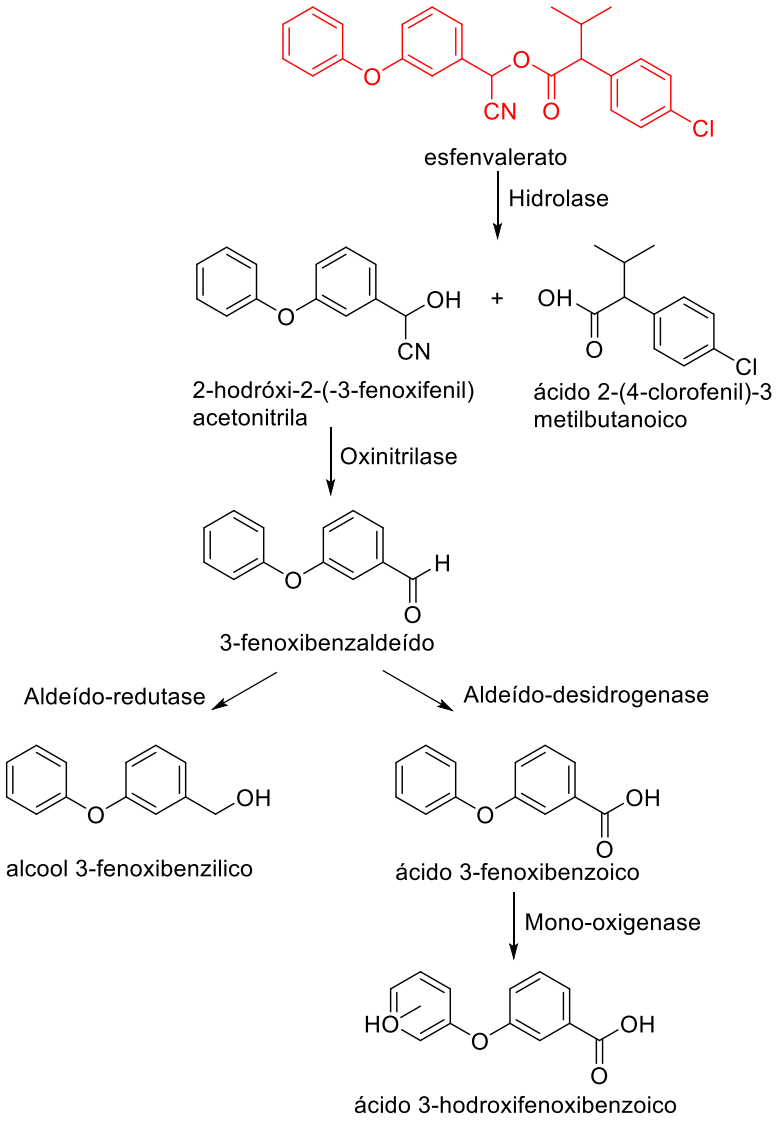


Fonte: Adaptado de Alarcón et al. (2014).

Além disso, a técnica de biorremediação pode ser utilizada em ambientes como solos e águas contaminadas utilizando a microbiota nativa com potencial de metabolização do poluente (ANDRADE et al. 2010).

Na agricultura, diversos ensaios de biodegradação já foram realizados. Estudos feitos por Birolli (2013) constataram a biodegradabilidade do pesticida piretróide esfenvarelato por fungos, determinando metabólitos mais polares, aumentando a possibilidade de carreamento desses compostos pela água.

Esquema 5. Proposta de rota biodegradativa do esfenvarelato a partir dos metabólitos detectados.



Fonte: Adaptado de Birolli (2013).

Testes recentes de biodegradabilidade foram desenvolvidos por Khan (2017) com poliuretano de poliéster utilizando o fungo *Aspergillus tubingensis.* Foi assumido que a alta biodegradação do poliuretano deve-se à disponibilidade de nutrientes, alto crescimento e secreção enzimática nas placas de cultura. O processo foi altamente dependente do pH, temperatura e fonte de carbono (no meio). A descoberta deste trabalho pode ser otimizada para biodegradação em grande escala de poliuretano de poliéster e outros polímeros.

É importante ressaltar que condições climáticas desfavoráveis, como elevadas ou baixas temperaturas, baixa umidade, acidificação do solo tendem a comprometer a degradação microbiana (PERISSINI, 2015).

Além do mais, a biodegradação é também citada em estudos recentes para corantes, petróleo, ácidos, herbicidas, efluentes têxteis e remoção de fármacos e desreguladores endócrinos do ambiente.

1.3.1. Biodegradabilidade de antibióticos e outros fármacos

Os antibióticos foram recentemente investigados como fonte de contaminantes ambientais emergentes. Essas substâncias podem exercer pressão seletiva que favorece bactérias resistentes (SCHWARTZ et al. 2003), que são uma grande preocupação de saúde pública.

Devido a crescente preocupação com a presença dos antibióticos e outros fármacos no ambiente, o número de pesquisas nos últimos 10 anos aumentou consideravelmente, especialmete no que se diz respeito às alternativas ecológica e economicamente viáveis para a remoção e descarte dessas substâncias que são consideradas microcontaminantes.

Longhin (2008), divulgou um quadro contendo informações sobre excreção e biodegradabilidade de substâncias terapêuticos.

Tabela 3. Excreção e biodegradabilidade de alguns agentes de uso terapêutico.

SUBSTÂNCIA	RAZÃO DE EXCREÇÃO (%)		BIODEGRADABILIDADE
	Sem alteração	Metabólitos	
Consumo humano			
Amoxicilina	80-90	10-20	Sem dados
Ampicilina	30-60	20-30	Sem dados
Penicilina G	50-70	30-70	Parcialmente degradável
Penicilina V	~40	~60	
Eritromicina	>60		
Cloranfenicol	5-10	-	Sem dados
Clorotetraciclina	>70	-	t½ = 20 dias (solo)
Oxitetraciclina	>80	-	t½ = 20 dias
Sulfametaxazole	15	-	Persistente
Tetraciclina	80-90	-	Persistente

Fonte: Longhin (2008).

Estudos avaliaram a degradação de três tipos de antibióticos sulfamidas (SMX, SDM e SMZ) em lodo, revelando resultados importantes. A degradação microbiana demostrou ser um processo importante para a remoção desse antibiótico do ambiente. As bactérias *Acinetobacter* e *Pseudomonas* representaram as principais comunidades envolvidas na degradação de sulfamidas (YANG, 2016).

De acordo com Borges et al. (2011), que desenvolveu ensaios de biotransformação com fungos endofíticos e fitopatogênicos para omeprazol, 5-hidroxiomeprazol e omeprazol sulfona, revelou que principalmente a linhagem de fungos fitopatogênicos estudados foram eficientes para a biotranformação do omeprazol e omeprazol sulfona obtendo metabólitos.

Os medicamentos veterinários são comumente usados para tratar numerosas doenças em animais. Os antibióticos constituem um dos grupos destes produtos farmacêuticos, sendo utilizados não só para o tratamento e prevenção de doenças, mas também para a promoção do crescimento e melhoria de animais (CROMWELL, 2002). O uso excessivo de medicamentos veterinários contribuiu para o surgimento desses produtos em vários compartimentos ambientais (LOKE, et al. 2000).

A partir da problemática emergente do uso excessivo de antibióticos veterinários, Alexandrino et al. (2017) desenvolveram uma pesquisa de biodegradação dos antibióticos enrofloxacina (ENR) e cefitiofur (CEF) utilizando uma comunidade microbiana associada. Neste estudo, ENR e CEF foram degradadas em diferentes extenções por comunidade microbiana para tratar águas residuais contaminadas com vestígios dos dois antibióticos. Os autorem concluiram que os locais contaminados com misturas desses antibióticos são passíveis de serem recuperados pelo processo de biorremediação.

2 OBJETIVOS

2.1. OBJETIVO GERAL

Realizar estudos de biodegradação do antibiótico cloranfenicol por fungos filamentosos endófitos isolados de *Bertholletia excelsa* (Castanha-do-Brasil).

2.2. OBJETIVOS ESPECÍFICOS

- Avaliar a interferência do cloranfenicol no crescimento micelial dos isolados fúngicos.
- Quantificar a biodegradação do cloranfenicol por fungos endofíticos;
- Analisar a natureza química dos metabólitos produzidos pela biodegradação;
- Realizar ensaios de toxicologia ambiental do cloranfenicol e dos produtos da biodegradação.

3 ARTIGO

Study of microbial degradation chloramphenicol by endophytic fungi isolated from *Bertholletia excelsa* (Brazil nuts)

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Abstract

Chloramphenicol (CAP), the compound aproached in this study, are a micropollutants and resists to conventional residual water treatment procedures. Thus, the biodegradation process employing specific and efficient microorganisms, including fungi, is an ecologically viable and low-cost option. Therefore, the aim of this study was to assess CAP biodegradability by five endophytic fungi strains isolated from *Bertholletia excelsa* collected in the Brazilian Amazonia. For this, the fungi strains were screened in solid/liquid medium and experimental design was performed to optimize culture conditions. In addition, an environmental toxicology assessment was carried out using the algae *Chlorella vulgaris*. Results from fungi cultures in solid medium demonstrated that CAP affected the strains growth and interfered in the development of conidia and spores. Moreover, the initial biodegradation screening showed that all strains managed to increase this antibiotic's degradation; *Trichoderma sp.* (BIORG 7), which was the strain that presented better results, was subjected to experimental design (*Box-behnken*) consisting of 15 experiments, having as variables: pH (5, 7, and 9), period (24, 48, and 72 hours), and CAP concentration (50, 100, and 150 $mg.L^{-1}$), reaching a biodegradation yield (by HPLC-UV) of 30% (24h, pH 7,0 and 150 $mg.L^{-1}$). The experimental design showed that the concentration has greater influence in the biodegradation process of the CAP by endophytic fungi. The metabolite 4-nitrobenzaldehyde was identified as a biodegradation product (by CG-MS) and product of biodegradation showed to be higher ecotoxicity in green algae. This metabolite that may be related with the diseases caused in different organisms.

Keywords: Micropollutants; Brazilian nut; Antibiotic Biodegradation; Plant-microorganism; Phenicol antibiotics; Environmental toxicity.

Introduction

Pharmaceutical compounds constitute a very important category of emerging micropollutants, which are considered a major risk to ecosystems due to their harmful biological effects (Miran et al. 2018)(Thelusmond et al. 2018). In addition, the most frequently microcontaminants detected in aquatic environments are drugs such as analgesics, antibiotics, lipid regulators, anti-inflammatories and synthetic hormones (Santos et al. 2010).

Antibiotics have been investigated as emerging environmental contaminants, since these compounds can contributes for the development of resistant bacteria, which are a major issue of public health due to the increased occurrence of clinical infections (Yang et al. 2016).

A broad-spectrum antibiotic that has been widely used is chloramphenicol (CAP), although this drug can be carcinogenic and genotoxic for humans (Liang et al. 2013). This drug is a liposoluble compound that diffuses through the cell membrane and reversibly binds to the 50S protein subunit of the prokaryote cell ribosomes, preventing the transfer of amino acids to the peptide chains in formation and consequently inhibiting the synthesis of proteins (Martins et al. 2018). The biotoxicity of nitro and chlorine groups this compound are responsible by bacterial activity, what resistant to conventional processes of biological wastewater treatment (Guo et al. 2017).

Many physical-chemical methods has been reported in the literature to the degradation of CAP, such as thermal (Tian and Bayen 2018), photocatalytic (Amildon Ricardo et al. 2018) (Chatzitakis et al. 2008) and electrochemical (Sun et al. 2017) treatment processes. However, although of the chemical structure simple, there are few reports of aerobic biological processes (biodegradation) by fungus, bacterium or microalga species.

Biodegradation processes are an environmentally friendly and represent a low cost option for micropollutants treatment (Alvarenga et al. 2014; Birolli et al. 2018), in which microorganisms use this compound as substrate by the action of their enzymes, converting

pollutants into nutrients and energy source for their survival (Le Borgne et al. 2008; Mouele et al. 2015).

However, complete biodegradation or mineralization involves the oxidation of intermediate compounds that can be more or less toxic substances than the starting compound (Serrano-González et al. 2018). These processes have several advantages when compared to physical methods, including low energy employment and levels of sludge production. The biodegradation of organic pollutants by fungi has been successful employed and described (Alvarenga et al. 2014; Vacondio et al. 2015).

Endophytic fungi, are promising biocatalysts, can survive in the tissues of healthy plants without causing any distinct infection in the host (Afzal et al. 2014). Additionally, studies have proven that entophytic fungi can also be used in the degradation of organic compounds (Potin et al. 2004), i.e., degradation of polycyclic aromatic hydrocarbons (PAHs), a class of toxic environmental pollutants. In another study, a strain of *Ceratobasidum stevensii* isolated from the *Eupharbiaceae* plant, removed 89.51% of phenanthrene (Dai et al. 2010). In addition, different works showed that *P. liquidambari*, isolated from aerial parts of *Bischofia polycarpam (Chen et al. 2013),* degraded organic pollutants efficiently, such as 4-hydroxybenzoic acid, ferulic acid, cinnamic acid and sinapic acid (Fu et al. 2018).

The interest in medicinal products derived from higher plants has increased significantly worldwide. The *Bertholletia excelsa* nuts popularly known in portugues as "castanha-do-brazil" (Brazil nut) are produced by a large tropical forest tree of the family Lecythidacea that grows throughout the Amazon Basin of South America (John and Shahidi 2010). It is noteworthy that the almond consists of 60-70% fat, 15-20% protein of good biologic quality, liposoluble Vitamins (A, E), and minerals (Ca, Fe, Zn, Na, K and Se) (Muniz et al. 2015).

Thus, considering this scenario, the objective of this study was to explore for the first time the biodegradability of the CAP antibiotic by endophytic fungi isolated from *Bertholletia excelsa*, collected in the Brazilian Amazon rainforest. Additionally, the influence of the pH, time and concentration of antibiotic in the rates of biodegradation were also assessed employing an experimental design.

Materials and methods

Reagents and solvents

The antibiotic chloramphenicol (98%) was obtained from Vetec®. Salts, reagents and solvents were obtained from Synth® and AppliChen Panreac®. Malt extract and Agar were purchased from Kasvi (Brazil) and isopropanol (HPLC grade) and acetonitrile (HPLC grade) were obtained from PANREAC and TEDIA.

Isolation of endophytic fungi from *Bertholetia excelsa*

The seed of *Bertholetia excelsa* (Brazil nuts) were collected by Brazilian Agricultural Research Corporation – Amapá, Brazil, at the localization area 1 - W 52°18'20,976" and S 0°33'44,44", and 2 - W 51°57'53,338" and S 0°25'21,39" (Amapá State, Brazil). The strains were isolated from the seeds according to the protocol described by (Azevedo, 1998) and stored according to (Kelly et al. 2017) at the Laboratory of Biocatalysis and Applied Organic Synthesis (Unifap). Theses seeds were washed thoroughly in distilled water, following the sterilization method described by (Barnet 1998), subsequently the surface of each seed and shells was sterilized with ethanol 70% for 1 minute, then a mixture of sodium hypochlorite 2% for 30 minutes and again rinsed with ethanol 70% for 30 min. After that, the seeds were rinsed three times with sterile distilled water. Then, the almonds and the shells were transferred to Petri dishes with filter paper moistened with sterile distilled water and incubated

in B.O.D at a temperature of 28°C. The plates were evaluated daily until the development of fungal colonies.

Identification of endophytic fungi strains from *Bertholetia excelsa*

Fungal morphology was investigated by direct observation through an optical microscope (OLYMPUS® BX41) and by squash mounts stained with Cotton Blue under a light microscope. Initial identifications were based on these observations and morphological criteria (Visagie et al. 2014). For this biodegradation study, the employed fungal strains were: *Aspergillus* sp. BIORG 4, *Aspergillus* sp. BIORG 5, *Penicillium* sp. BIORG 6, *Trichoderma* sp. BIORG 7, and *Aspergillus* sp. BIORG 9.

Growth of strains in the presence of the antibiotic chloramphenicol

Solid culture media (2% malt) at pH 7 were prepared and sterilized in autoclave (Phoenex) at 121ºC for 20 min. Next, a CAP solution (125 mg solubilized in 5 mL of DMSO) was added to the media at a final concentration of 100 $mg.L^{-1}$ in the Petri dishes, the addition was performed at 40-50ºC to prevent thermal degradation (Vacondio et al. 2015). Subsequently, the medium was homogenized with gentle circular movements. After 24 h, the Agar plates were inoculated with a central insertion point and incubated at 28ºC (B.O.D., LUCADEMA®, model LUCA – 161/03) for 9 days and monitored every each 24 h. Solid medium plates without the CAP were used as fungal control.

Biodegradation of chloramphenicol in liquid medium (Initial Screening)

In a Erlenmeyer flasks (125 mL) containing 50 mL of malt liquid medium (2%) at pH 7.0 were employed for fungi cultivation. The inoculations were carried out with seven circular slices (0.5 cm diameter) from solid cultures incubated for 5 days at 32 ºC (B.O.D.). Next, the

culture was incubated in orbital shaker for 5 days (32ºC, 130 rpm) and then 100 mg L^{-1} of CAP (previously dissolved in 5 mL DMSO) were added. In addition, the reactions were incubated in orbital shaker for 3, 6 and 9 days (32 ºC, 130 rpm) and all biodegradation experiments were performed in triplicate.

Biodegradation of chloramphenicol through Experiment design and statistical model

In this study, a three level and three variable Box–Behnken factorial design was applied to determine the best combination of factors for CAP biodegradation employing the selected endophytic fungus (*Trichoderma* sp. BIORG 7). Reaction time (h), pH of the medium and CAP concentration (mg.L^{-1}), which were identified to have strong effects on the response in preliminary one-factor-at-a-time experiments, were taken as the variables tested in a 15-run experiment to determine their optimum levels. Independent variables were designated as *x1*, *x2* and *x3*, and their levels values are shown in Table 1. The polynomial equation used for the three variables is described in the Equation 1

$$Y = \beta 0 + \beta 1x1 + \beta 2x2 + \beta 3x3 + \beta 11x1^2 + \beta 22x2^2 + \beta 33x3^2 + \beta 12x1x2 + \beta 13x1x3 + \beta\ 23x2x3 \quad [Eq.1]$$

Where: *Y* is the predicted response; *β0* is the model constant; *β1, β2*, and *β3* are the linear coefficients; *β11, β22*, and *β33* are the quadratic coefficients; *β12, β13*, and *β23* are the interaction coefficients; and *x1*, *x2*, and *x3* are independent variables (Dong et al. 2009).

Table 1 Three independent variables used in the employed Box-Behnken factorial design.

Factor	**Name**	**Levels**		
		-1	**0**	**+1**
X_1	Time (h)	24	48	72
X_2	pH of the medium	5	7	9
X_3	Chloramphenicol concentration (mg.L^{-1})	50	100	150

The software STATISTICA® (version 10, Statesoft – Inc., Tulsa, USA, trial version, 2011) was used for experimental design and data analysis. Analysis of variance (ANOVA) was used for evaluation of independent variables significance, influence and interactions. A Pareto charts was produced to the obtainment of the significance of the tested variables on the mentioned responses.

Extraction of chloramphenicol and its metabolites

After the biodegradation reaction, the fungal mycelia were filtered on a Buchner funnel into a 250 mL Kitasato flask, then the pH of the culture broth was measured for adjustment and standardization at 7.0 (QUALXTRON®). The filtered mycelia were suspended in 50 mL of water and ethyl acetate (1:1) and kept under vigorous magnetic stirring for 30 min. This procedure promotes the cell lysis and extraction of the CAP content adsorbed and absorbed by the fungal strains, since the method validation showed that this methodology was appropriated. After that, the suspension was filtered into a Buchner flask together with the culture broth and the sample was extracted with ethyl acetate in three steps (3 × 30 mL), dried with anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residue was dissolved in methanol in a 5 mL volumetric flask.

Method validation

To validate the developed method, 2% Malt culture medium was prepared in five 125 mL-Erlenmeyer flasks containing 50 mL of culture medium. In each of them, 7 circular fragments (5 mm) of the fungal strain BIORG 7 were inoculated, and the experiment was placed in an incubator for orbital shaking for 5 days, 32 °C and 130 rpm. Thereafter, the reactions were sterilized in autoclave for 20 minutes at a temperature of 121° C for death of

the fungal cells and inactivation of the enzymes. Then, the CAP concentration was added (100 mg L^{-1}) and the samples were extracted as described in previous section.

It was also performed killled-Cells controls, which were prepared as the experiments for method validation. However, after sterilization and antibiotic addition, the sample was placed in an orbital shaker for 76 h at 32 °C and pH of 6.6 as in the optimal conditions. Then, the samples were prepared for chromatographic analysis (Next section).

Quantification of chloramphenicol by HPLC-UV analysis

CAP was quantitatively determined by High Performance Liquid Chromatography (HPLC) using a Shimadzu chromatographic system constituted by the following modules: LC-20 AT pumping system, DGU-20A5 degasser, SIL-20AHT automatic sampler, detector UV-VIS SPD M20A, CTO-20A column oven and CBM-20A system controller. Separations were performed using a Phenomenex C18 Luna Column (5μm of particle size, 25 cm x 4.6 mm). The material was eluted using a mixture of water (solvent A) and acetonitrile (solvent B) and, as follows: isocratic (solvent B), 0-19 min, 60%; 19-20 min, 60-90%; 20-35 min, 90%; 35-36 min, 90-60%; 36-45 min., 60% The temperature of the oven was 40 ° C, flow of 0.7 $mL.min^{-1}$ and injection volume of 10 μL. The ultraviolet detection was performed at 277 nm.

To determine the CAP concentration, the external standard method was used, resulting in a equation: $c = Ax + B$.

Where c = analyte concentration in mg L^{-1};

x = area in the analyte;

A = angular coefficient;

B = linear coefficient.

It is important to note that the samples were suspended in 5 mL of methanol after the liquid-liquid extraction, so the samples were concentrated 10-fold. Therefore, standard solutions of 50, 350, 650, 950 and 1250 mg L^{-1} in methanol were employed for the quantification of CAP, generating the linear equation: c = 26277.x + 187773.

Detection of metabolites by CG-MS analysis

The analyzes for the metabolites detection were performed by gas chromatography coupled to mass spectrometry in a Shimadzu / GC-2010 apparatus equipped with Shimadzu / AOC-5000 auto injector and a Shimadzu MS2010 plus in SCAN mode, 70 eV. The chromatograph oven was equipped with a DB-5 fused silica column (J & W Scientific, 30 m x 0.25 mm x 0.25 μm) with helium as carrier at 63 kPa.

The injector temperature was 250 ° C and the detector temperature was 280 ° C. The initial oven temperature was 110 ° C for 2 min and increased to 300 ° C with a heating rate of 20 °C min^{-1}, maintaining this temperature for 10 min and resulting in a total analysis time of 45 min. The split ratio was 1: 1.

Environmental toxicology test

The green alga *Chlorella vulgaris* isolated from water samples obtained from *Lagoa dos Índios*, located in the municipality of Macapá (0.031368 latitude and 51.102559 longitude) was used for environmental toxicity analysis of CAP and its metabolites. A serial dilution was performed to isolate the colony and the cells were inoculated into nitrogen / phosphorus / potassium (NPK) medium. An algae count was performed using a Neubauer chamber, in which a cell density of 1×10^4 cells ml^{-1} was employed for all tested groups in a 10 mL of *C. vulgaris* inoculum cultured in NPK, 8: 8: aqueous medium. The experiments were performed after 24, 48, 72 hours and 5, 10 and 15 days (Oliveira et al. 2017).

The concentrations of 50, 100 and 150 mg mL^{-1} of each substrate (biodegradation products, fungal metabolites, and CAP) were added to each sample of *C. vulgaris*. The experiments were performed in triplicate.

Results and discussion

Fungal growth on solid medium

Radial growth tests with the strains isolated from Brazil nut trees containing CAP at 100 $mg.L^{-1}$ or only malt at 2% as a control were performed for a preliminary assessment of the CAP effects on the endophytic fungi strains. The experiments were carried out for 9 days and the growth of the colonies were evaluated at every 24 hours, the results are shown in Table 2.

Table 2 Colony growth of the fungi strains isolated from Brazil nut in solid medium with central insertion point

Strains	Growth medium	Time (days)		
		3	6	9
		Colony diameter[a] (cm)		
Aspergillus sp. BIORG 4	Malt 2%	2.7±0.4	5.7±0,8	7.5±0.4
	Malt 2% + CAP	3.5±0.1	6.7±0,2	8.0*
Aspergillus sp. BIORG 5	Malt 2%	2.6±0.7	5.5±0,8	8.0*
	Malt 2% + CAP	2.9±0.1	5.6±0,1	8.0*
Penicillium sp. BIORG 6[b]	Malt 2%	1.7±0.1	2.8±0,1	3.7±0.1
	Malt 2% + CAP	1.2±0.1	2.4±0,1	3.5±0.1
Trichoderma sp. BIORG 7[c]	Malt 2%	8.0*	8.0*	8.0*
	Malt 2% + CAP	6.6±0.2	8.0*	8.0*
Aspergillus sp. BIORG 9[d]	Malt 2%	1.9±0.2	3.4±0,7	4.9±0.9
	Malt 2% + CAP	2.1±0.1	3.5±0,1	4.8±0.1

[a] The diameter of the colonies were evaluated up to 8.0 cm, since the Petri dishes used in this study had 9.0 cm of diameter. [b] The colony of this strain reached 8.0 cm of diameter after 21 days (malt at 2%) and 22 days for the control experiment (malt at 2% + CAP). [c] The diameter of the colony strain was performed every 24 hours due to its fast growth. [d] The colonies (malt at 2% and malt at 2% + CAP) reached the maximum diameter of 8.0 cm after 11 days of growth.

For the strain *Aspergillus* sp. BIORG 4, the presence of CAP induced a slight increase of the colony diameter, when compared to the control experiment containing only malt at 2%. Suggesting that this fungus may use this antibiotic as a source of carbon, hence inducing mycelial growth. While the strain reached 8.0 cm diameter in the eighth day in the presence of CAP, the control colony reached this value in the tenth day.

In the experiments employing the strains *Aspergillus* sp. BIORG 5, *Penicillium* sp. BIORG 6 and *Aspergillus* sp. BIORG 9 there were no significant differences of the mycelial diameter in the presence and absence of CAP, which suggest that antibiotic does not interfere on their growth. However, it is important to emphasize that this result is not conclusive for

longer periods of exposure. These three strains presented differences of color between the incubation in the presence of CAP during the growth experiment (Table 2).

The *Trichoderma* sp. BIORG 7 strain presented significant inhibition of growth in the presence of CAP in the second and third days, when compared to the control experiment (Table 3). There was a noticeable difference of color in this strain during the experiment (Table 4).

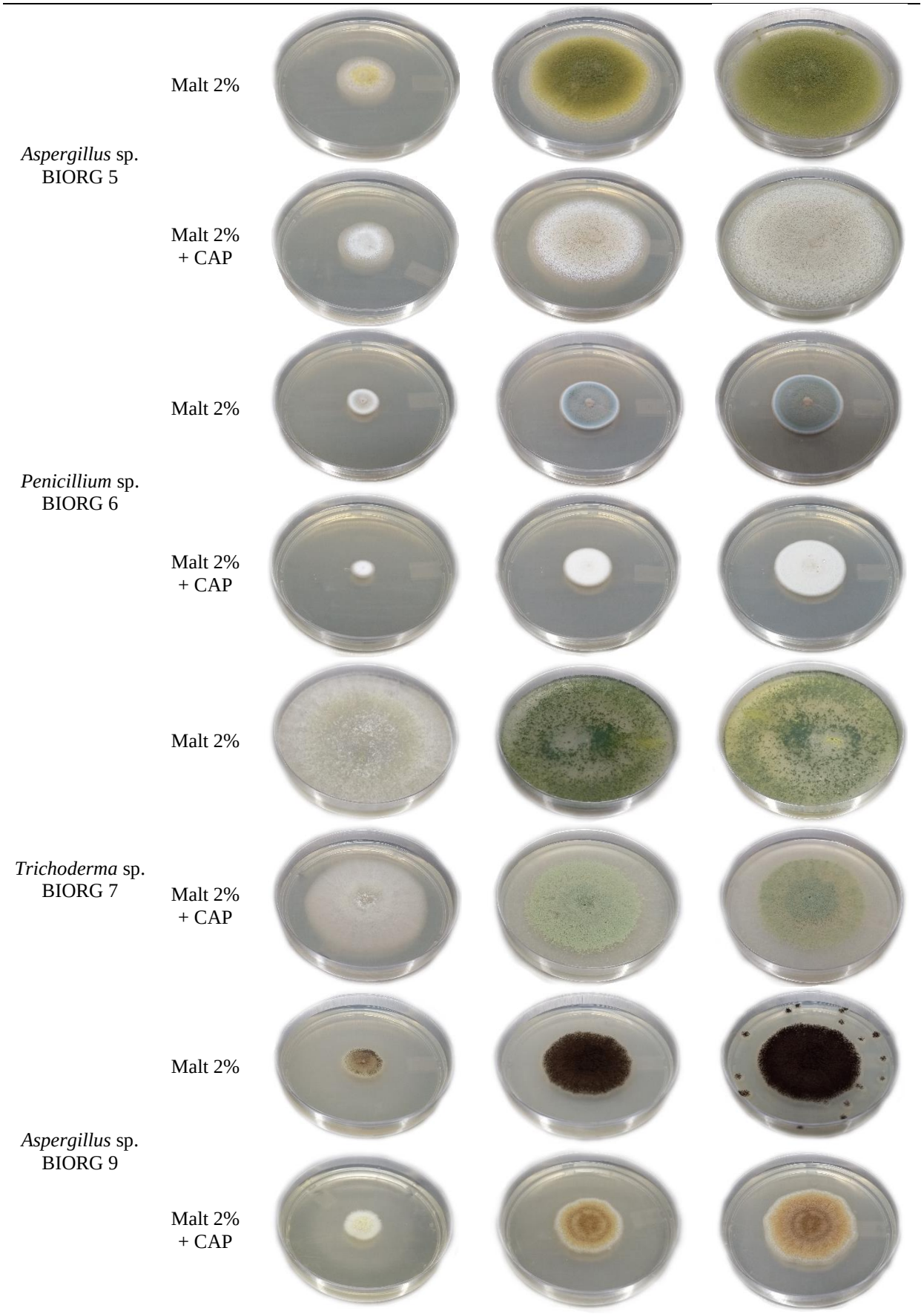
Table 3 Mycelial growth of *Trichoderma* sp. (BIORG 7) in solid medium with central insertion point

Strains	**Growth medium**	**Time (days)**		
		1	2	3
		Colony diameter[a] (cm)		
Trichoderma sp. BIORG 7	Malte 2%	1.6±0.1	6.5±0.2	8.0[a]
	Malte 2% + CAP	1.3±0.2	3.9±0.2	6.6±0.2

[a] The diameter of the colonies were evaluated up to 8.0 cm, since the Petri dishes used in this study had 9.0 cm of diameter.

Table 4 Microbial growth experiments for isolated Brazil nut strains for the study of CAP biodegradation

Strain	**Growing medium**	**Colony Growth**		
		3 days	6 days	9 days
Aspergillus sp. BIORG 4	Malt 2%			
	Malt 2% + CAP			


Malt 2%
Aspergillus sp. BIORG 5
Malt 2% + CAP
Malt 2%
Penicillium sp. BIORG 6
Malt 2% + CAP
Malt 2%
Trichoderma sp. BIORG 7
Malt 2% + CAP
Malt 2%
Aspergillus sp. BIORG 9
Malt 2% + CAP

For all the endophytic fungi (*Aspergillus* sp. BIORG 4, *Aspergillus* sp. BIORG 5, *Penicillium* sp. BIORG 6, *Trichoderma* sp. BIORG 7, and *Aspergillus* sp. BIORG 9), incubated in solid medium in the presence of CAP was observed the conidiogenesis process and consequently a decrease in the production and retardation of spore maturation, but with no effect on growth. The prerequisite conditions for sporulation as well as secondary metabolism are generally specific more than those conditions that permit vegetative growth (Sekiguchi et al. 1977).

Similarly, was observed in the fungitoxi fungitoxic effects of Imidacloprid and Fipronil on *B. bassiana* and *M. anisopliae* fungi, causing a decrease in conidia production. Moino & Alves (1998) Too it was reported the interference in the conidia production and fungal growth through the action of pesticides and fungicides (thiophanate methyl, cartape, methyl parathion, tebuconazole and tetraconazole) in strains of entomopathogenic fungi (Loureiro et al. 2002).

This inhibition could be a result of a nocive effect caused by the antibiotic, or due to some metabolite formed during the antibiotic metabolism. Further experiments revealed that this inhibition was due to the production of 4-nitrobenzaldehyde or other metabolic as biodegradation products, which is toxic for microorganisms in general.

Method validation

The quintuplicate performed for method validation presented a CAP concentration of 98.8±1.6 mg L^{-1}, representing 98.8% of accuracy and 1.6% of standard deviation. Moreover, the value of the standard deviation of the samples demonstrates the precision of the developed method. However, it is important to note that the results of the CAP biodegradation tests (BD%) had the interference of the metabolite obtained by the biotransformation of this

substrate, the toxic 4-nitrobenzaldehyde to the microorganisms. In this way, the proposed method has great applicability in other substrates of interest for biodegradation.

Selection of microorganisms in liquid medium for biodegradation

All the five endophytic strains of fungi employed in this study were able to grow in the presence of CAP. Hence, culture in liquid medium was performed to evaluate each fungi efficiency in the biodegradation of this antibiotic.

The strains were cultured over 5 days in a liquid medium in orbital stirring in malt 2% containing 100 $mg.L^{-1}$ of CAP. Biodegradation reactions occurred for, 6 and 9 days. These results indicate that all microorganism samples tested increased the biodegradation of CAP since its residual concentrations were lower than that determined for the control groups and method validation.

Data from Table 5 show that all strains could increase CAP biodegradation, specially the *Trichoderma* sp. BIORG 7 and *Aspergillus* sp. BIORG 9 (25.2% and 29.3% respectively). Based on biodegradations results and standard deviation, the strain *Trichoderma* sp. BIORG 7 was selected for further experiments in different periods of biodegradation, pH and concentration of CAP, in order to optimize its degradation efficiency.

Table 5 Residual concentrations and biodegradation percentual of CAP for the screening of endophytic fungi (32 °C, 130 rpm of orbital stirring, Initial concentration of 100 mg L^{-1})

	Time (days)	BIORG 4	BIORG 5	BIORG 6	BIORG 7	BIORG 9
Residual concentration of CAP (mg L^{-1})	3	91.5±2.3	78.9±1.4	90.1±9.5	76.7±0.3	70.7±20.1
	6	75.8±4.1	73.1±0.4	73.5±2.1	76±0.6	82.4±22.4
	9	74.7±2.2	75.4±3.6	76.9±4.7	74.8±4.2	85.9±3.3
CAP biodegradation (%)	3	8.5	21.1	9.1	23.3	29.3
	6	24.2	26.9	26.5	24	22.4
	9	25.3	24.6	23.1	25.2	14.1

[a] Abiotic control: 97.9±1.1.

Evaluation of chloramphenicol biodegradation through experimental design

Based on the best results of the previous section, CAP biodegradation content (BD%) was obtained from different trials of the experimental design protocol with the fungus *Trichoderma* sp. BIORG 7. Variables values are showed in Table 6. A Pareto chart of standardized effects presented in Fig 1 shows significant effect of CAP concentration ($mg.L^{-1}$), reaction time and pH of the medium variables (quadratic). The bar length of each parameter characterizes the absolute importance of the estimated effects. The vertical line represents the limit between the significant and insignificants effects with a 5% risk of error. Three effects are significant at 95% confidence level in the studied experimental domain ($P < 0.05$) as shown in Fig 1.

Table S1 (*Supplementary Information*) provides the ANOVA of the model. The value of the coefficient of determination (R^2) was 0.81. The proficiency of the model is demonstrated if R^2 is equal to 0.75 or higher than this value (Haaland, 1989). The response surface of the CAP biodegradation content as a function of pH of the medium (*x2*) and contact time (*x1*) is presented in Fig. 2 as a 3D response surface plot. Interactions between these two factors show that the reaction time does not interfere on the response.

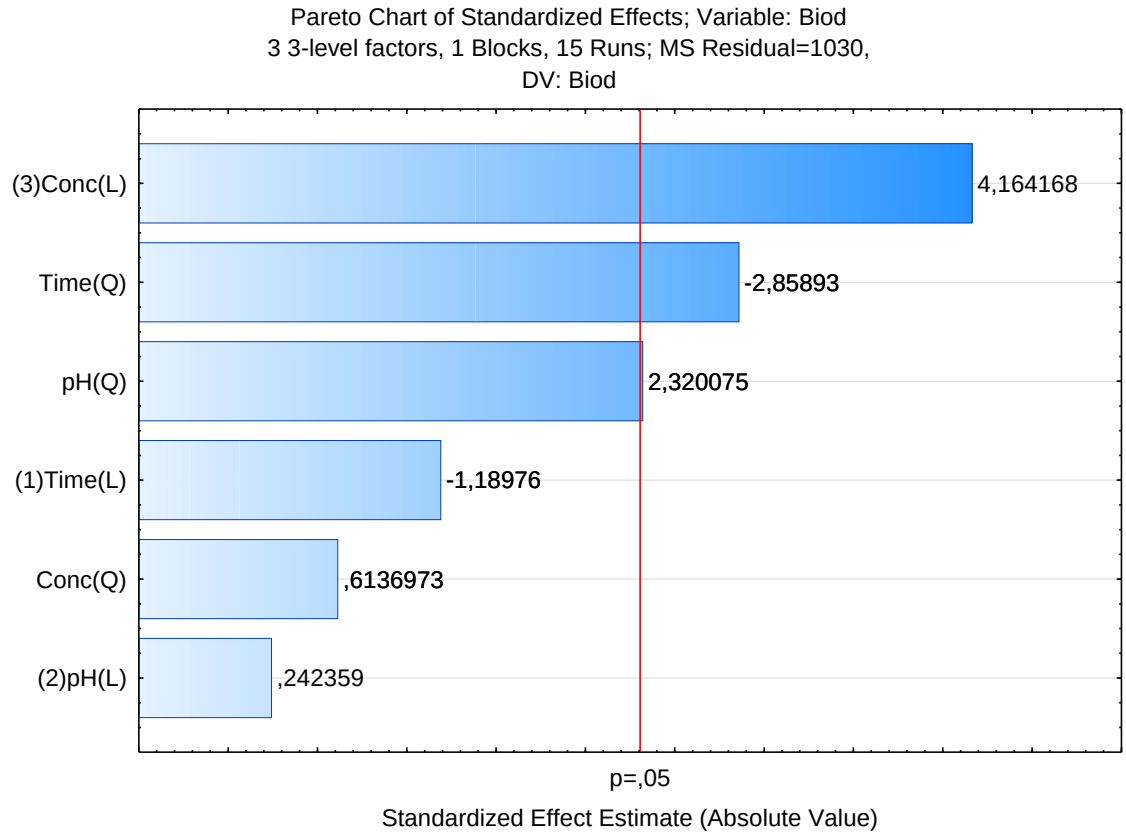


Fig. 1 Pareto chart of effects for the CAP biodegradation content (%)

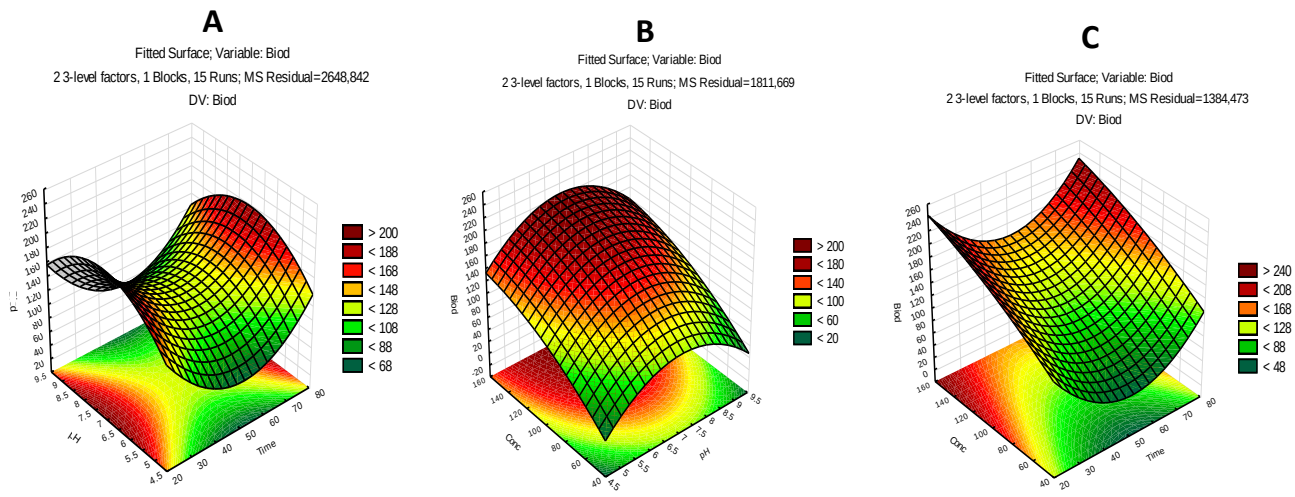


Fig. 2 Response surface plot and contour plot of the CAP biodegradation content as a function of (A) reaction time (*x1*) and pH of the medium (*x2*); (B) pH of the medium (*x2*) and CAP concentration (*x3*); (C) CAP concentration (*x3*) and contact time (*x1*)

On the other hand, Fig. 2 shows that an increase of CAP concentration (*x3*) had a great influence on the biodegradation content for any value of pH. The maximum CAP biodegradation content (24.3%) was obtained at the highest CAP concentration (150 ppm) and pH 7.

Recent study of Ma et al. 2019 showed an efficient consortium of bacteria (*Sphingobium* sp., *Pandoraea* sp., *Comamonas* sp., *Pseudomonas* sp. and *Cupriavidus* sp.) in the degraded of CAP, over 63% in 24 hours. However, filamentous fungi may have advantages in a biodegradation system of organic pollutants, because it has greater cell stability compared to prokaryotes (Hernández et al. 2017).

Chloramphenicol concentration (*x3*) and reaction time (*x1*) were statistically the most significant factors. According to the results presented in Figure 3, BD% increased when *x3* was increased from 50 to 150 ppm for 24 hours (Table 6). However, good results were also observed at 48h, although the most significant biodegradation rate was in the shortest experiment time (24h) with the highest concentrations of the antibiotic. The experimental design with 3 factors is more efficient for the definition of the best conditions and parameters, giving greater amplitude to the results (Collins et al. 2009).

Table 6 The design matrix and responses for the variables levels

Run	Uncoded and coded variables levels						Responses (BD%)
	x1		*x2*		*x3*		
1	24	-1	5	-1	100	0	13.6
2	72	1	5	-1	100	0	10.0
3	24	-1	9	1	100	0	18.0
4	72	1	9	1	100	0	16.4
5	24	-1	7	0	50	-1	13.2
6	72	1	7	0	50	-1	14.4
7	24	-1	7	0	150	1	24.3
8	72	1	7	0	150	1	17.5
9	48	0	5	-1	50	-1	3.6
10	48	0	9	1	50	-1	2.0
11	48	0	5	-1	150	1	18.1
12	48	0	9	1	150	1	11.1
13	48	0	7	0	100	0	13.6
14	48	0	7	0	100	0	13.6
15	48	0	7	0	100	0	13.6

Fig. 3 shows, through a chromatogram generated by HPLC-UV, the results of CAP biodegradation by the experimental design. The experiments show the peaks of CAP at a retention time of 19.3 minutes.

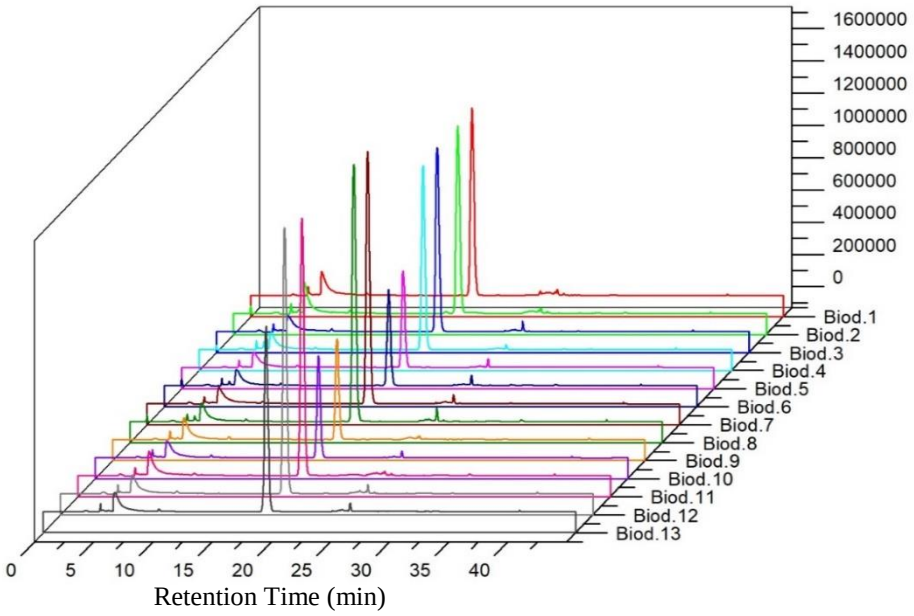


Fig. 3 Chromatogram of CAP biodegradation by HPLC - UV through experimental design

Identification of chloramphenicol metabolites

The biodegradation extract was analyzed by GC-MS for metabolites detection and identification. The 4-nitrobenzaldehyde was identified as a metabolite of the CAP biotransformation with a retention time of 19.3 min, this compound identity was confirmed later with a standard. It is important to note that 4-nitrobenzaldehyde was not present in the fungal control of the strains and neither in the analysis of the CAP standard at the same concentration.

Chloramphenicol has been associated with aplastic anaemia in humans and reproductive/hepatotoxic effects in animals without a clear mechanism of action. Therefore, it is possible that reactive metabolites such as 4-nitrobenzaldehyde presented in this biodegradation study with fungi may be involved in the disease cause mechanism in eukaryotic cells.

Environmental toxicology analysis

For environmental toxicology assessment, 10 mL of an inoculum containing *Chlorella vulgaris* cultivated in liquid NPK medium was collected (08:08:08), with 7.5 mL of NPK for each liter of distilled water. The density of cells was determined employing in a Neubauer chamber for inoculum padronization. In the experiments with enzymatic broth containing the biodegradation products of CAP, a slight decrease of cells number compared to the initial amount was observed in the first 24 hours, mainly at concentrations of 100 and 150 $mg.L^{-1}$ (0.73 and $0.72x10^6$ cell $m.L^{-1}$, respectively). After that, the number of *Chlorella vulgaris* cells remained stable with variations of $0.55x10^6$ to $1x10^6$ in the three concentrations until the tenth day of the experiment. Surprisingly, in the last cell counting occurred a significant decline of population in all concentrations – $0.15x10^6$ (50 $mg.L^{-1}$), $0.25x10^6$ (100 $mg.L^{-1}$), and $0.3x10^6$

(150 mg L^{-1}) cell mL^{-1}, indicating a toxic effect on the algae caused by the metabolites produced during the CAP biodegradation (Fig. 4).

Regarding the experiments performed only with the metabolites produced by the fungi (without CAP), a decreased concentration of cells was observed, mainly in the last five days of the experiment. In the first 24 hours, there was a slight increase in cells quantity for the concentrations 50 and 100 $mg.L^{-1}$. Then, an abrupt increase of cellular density occurred in the concentrations 100 and 150 $mg.L^{-1}$ of fungi metabolites. From then on, the values of density regressed; in the last day, the values were 0.33 for 50 $mg.L^{-1}$, 0.3 $mg.L^{-1}$ for 50 $mg.L^{-1}$, and $0.33x10^{6}$ for 150 $mg.L^{-1}$. This indicates that the metabolites produced by the fungi moderately affect algae's rate of growth.

Recent studies have shown that the toxics effects of the CAP in some species of algae (*Pseudokirchneriella subcapitata*, *Scenedesmus quadricauda*, *Scenedesmus obliquus* e *Scenedesmus acuminatus*), promoting negative effects on the growth and alteration of biochemical components, with the composition and structure of lipids, proteins and DNA (Xiong et al. 2018).

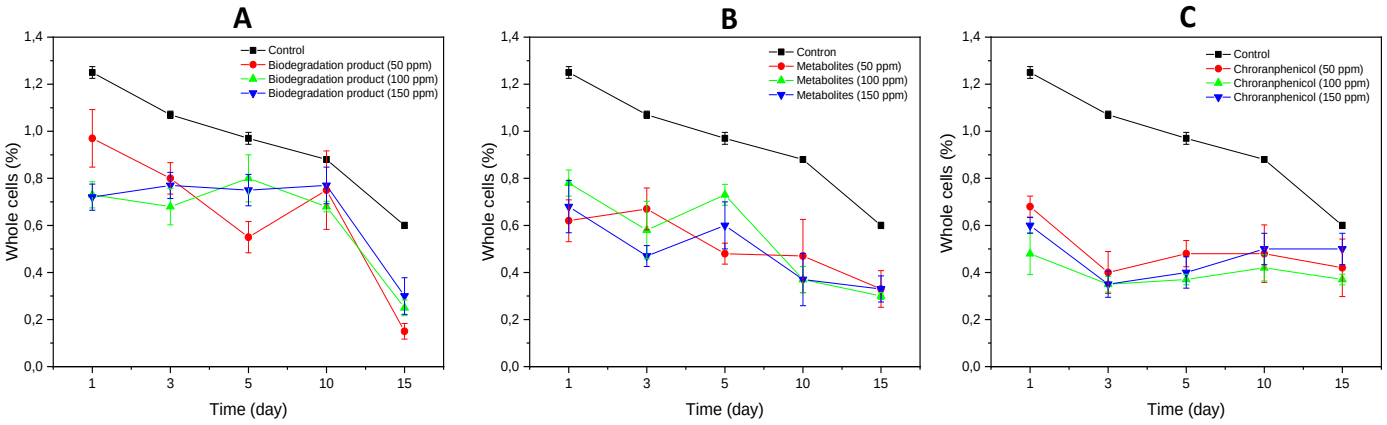


Fig. 4 *Chlorella vulgaris* number of cells for toxicity evaluation A) CAP biodegradation products; B) Fungal metabolites; C) Chloramphenicol solution

In the experiments with CAP solution alone, the results show pronounced decrease of cell quantity in all concentrations: from $0.68x10^6$ to $0.4x10^6$ (50 mg L^{-1}), from $0.48x10^6$ to $0.35x10^6$ (100 mg.L^{-1}), and from $0.6x10^6$ to $0.35x10^6$ cell.mL-^{1} (150 mg L^{-1}). Overall, the solution of CAP led to a decrease in the cell quantity when compared to the control group, it is important to note that this solution presented higher environmental toxicity after the third day in contact with *Chlorella vulgaris*.

Conclusion

This was the first study that showed the use of endophytic fungi in the biodegradation process of the micropollutant CAP. The strains *Aspergillus* sp. BIORG 9 and *Trichoderma* sp. BIORG 7 presented better results of biodegradation, 29.3% in 3 days and 25.2% of biodegradation in 9 days, respectively. The experimental design applied to the strain *Trichoderma* sp. BIORG 7 was fundamental for the optimization of the experimental conditions. Reducing the employed resources as described for in the green chemistry principles, since the maximum experimental period was of 72 hours, using 15 samples only.

Through statistical analysis, it was possible to conclude that the concentration of CAP was the factor with the highest influence over biodegradation, and the second most important factor was the time. In brief, the best biodegradation conditions with the selected microorganism was 24 hours, pH 7, and CAP concentration of 150 mg.L^{-1}. This study showed that endophytic fungi, including *Trichoderma* sp. BIORG 7, showed potential to be important biocatalysts for future green processes and can be able to improve the biodegradation of other contaminants, including different approaches as the employment of microbial consortia and other associations.

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Supplementary Information

Study of microbial degradation chloramphenicol by endophytic fungi isolated from *Bertholletia excelsa* (Brazil nuts)

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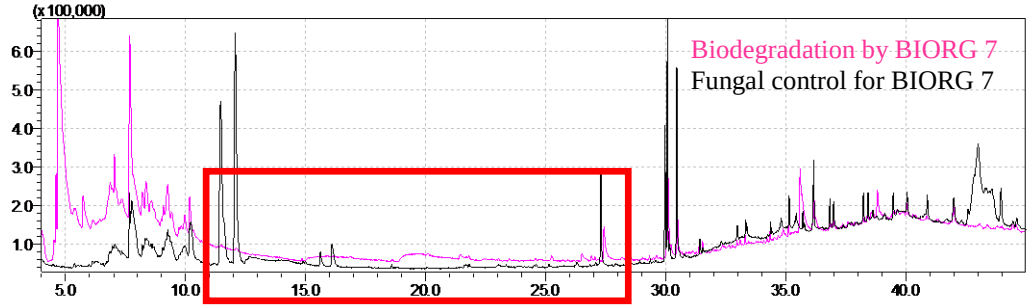
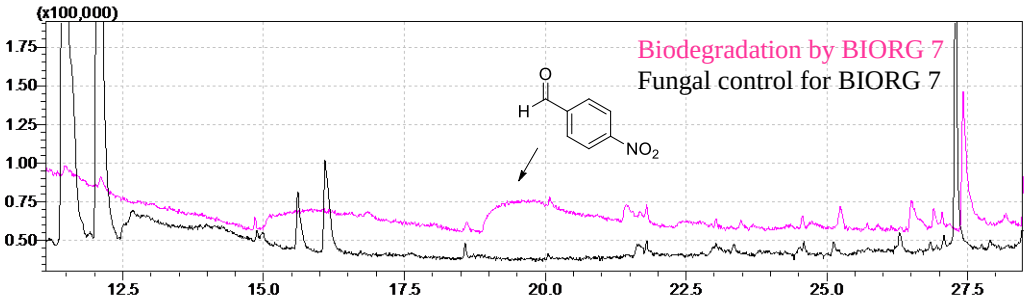
Spectral data

Figure S1. A) GC-MS chromatogram of chloramphenicol biodegradation by BIORG 7 in optimized conditions (24 h, pH 7.0, 32 °C, 130 rpm). B) Expansion between 11.0 and 28.0 min.

A)



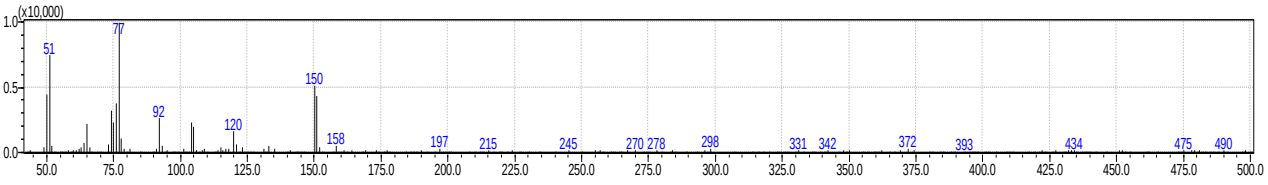
B)



Spectrum Analysis

Figure S2. MS spectra of A) 4-nitrobenzaldehyde in the biodegradation experiment by BIORG 7 (24 h, pH 7.0, 32 °C, 130 rpm) and B) compound standard.

A)



B)

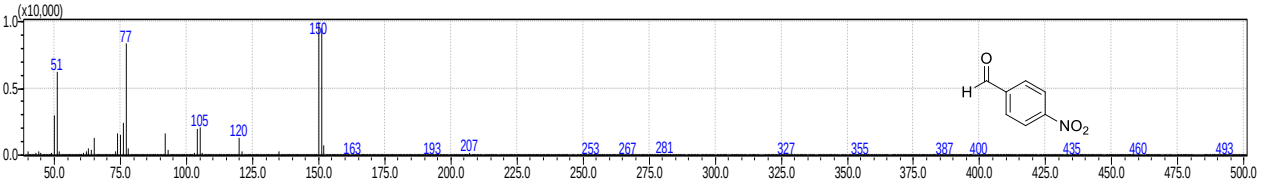


Table S1. Analysis of variance (ANOVA) of the model.

Sources	**SS**	**Df**	**MS**	***F*-value**	$F_{0.05}$
Biodegradation yield					
*X*1	1458.00	1	1458.00	1.41553	0.268249
*X*2	60.50	1	60.50	0.05874	0.814600
*X*3	17860.50	1	17860.50	17.34029	0.003147
$X1^2$	8418.69	1	8418.69	8.17349	0.021186
$X2^2$	5544.23	1	5544.23	5.38275	0.048914
$X3^2$	387.92	1	387.92	0.37662	0.556459
Pure error	8240.00	8	1030.00		
Total	43165.33	14			
R-squared 0,81					

SS: *sum of squares,* Df: *degrees of freedom,* MS*: mean square*

4 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

As pesquisas em biocatálise têm demostrando nas ultimas décadas ser um egresso para as Ciências Farmacêuticas na busca por novas moléculas bioativas. As técnicas biocatalíticas, se bem executadas são bastante eficientes em termos quantitativos e qualitativos, e estão em consonância com os princípios da Quimica Verde. Dentro do referido contexto, a biodegradação se tornou uma alternativa viável para a remoção de substâncias microcontaminantes emergentes persistentes no ambiente, como é o caso dos fármacos, que dificilmente são retirirados do ambiente por técnicas convencionais de tratamento de efluentes. Além da possibilidade de investigação dos metabólitos prozudidos por esse tipo de xenobiótico que pode provocar sérios danos à saúde humana e ao próprio ecossistema.

A perspectivas para o futuro próximo devem estar centradas na investigação de outros substratos de interesse afim de buscar o aperfeiçoamento das técnicas de biodegradação e biotransformação e para o desenvolvimento de biorreatores com microrganismos endofítcos , devido sua evidente emergância científica. É importante destacar que está pesquisa se torna pioneira para o desenvolvimento biotecnológico da Amazônia brasileira, já que pela primeira vez foram usados fungos endofíticos isoados dessa região para o estudo de biodegrdação de um micropoluente. O fomento para pesquisas desse porte devem ser incentivadas, com o objetivo de solucionar os problemas dos micropoluentes emergentes, consequentemente a investigação de metabóltitos de interesse, traduzindo no aperfeiçoamento das técnicas de biocatálise, visto o grande potencial de biodiversidade que existe na regãos norte do Brasil.

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Anexo 1. Normas de Publicação da Revista / Jornal

Instructions for Authors

Types of Papers

- Peer-reviewed contributions:
  • Research Articles (full papers)
  • Short Original Communications and Discussion Articles
  • Review Articles
  • Research Communications
  Please ensure that the length of your paper is in harmony with your research area and with the science presented.
  All papers – excluding Editorials, Letters to the Editor, Conference Reports – are subject to peer-review by a minimum of two and a maximum of three experts.
  While submitting your paper you will be asked for three potential reviewers. Indicating three reviewers is mandatory.
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  - For local studies, please indicate the name of the region and country in the title.
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Please provide an abstract of about 10 to 15 lines.

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Please provide 6 to 8 keywords which can be used for indexing purposes

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- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
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Please use no more than three levels of displayed headings.

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Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

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Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

Citation

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995a, b; Kelso and Smith 1998; Medvec et al. 1999, 2000).

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

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- Journal article

  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8

  Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329
- Article by DOI

  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086
- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London
- 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, pp 230-257
- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007
- Dissertation

  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

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- ISSN LTWA

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For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

- EndNote style (zip, 2 kB)

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  wikipedia documents are not acceptable as references.
- Language
  References should be in English with an appropriate title in English. If it’s in a different language the language should be indicated
  Zhu J, Wu F-C, Deng Q-J, Shao S-X, Mo C-L, Pan X-L, Li W, Zhang R-Y (2009) Environmental characteristics of water near the Xikuangshan antimony mine. Acta Scientiae Circumstantiae 29:655-661 (in Chinese)
- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

Electronic Figure Submission

- Supply all figures electronically.

- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

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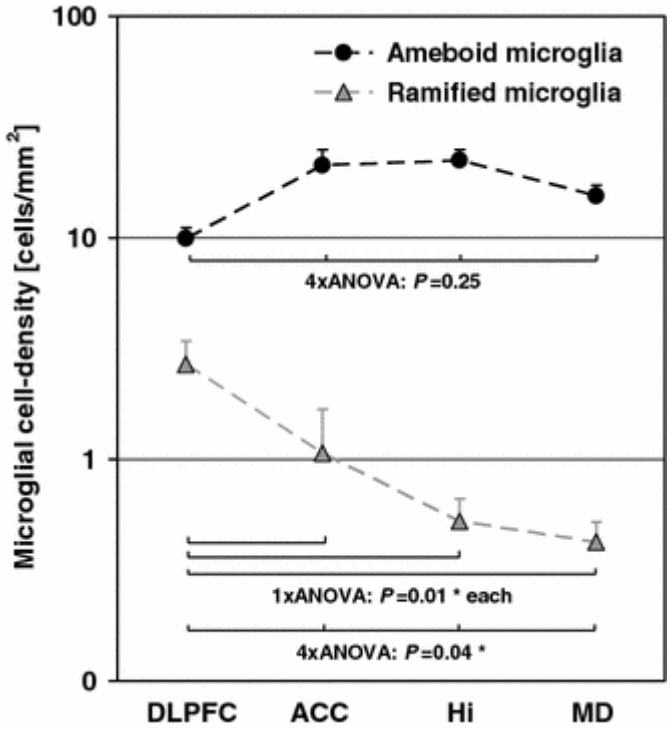

- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
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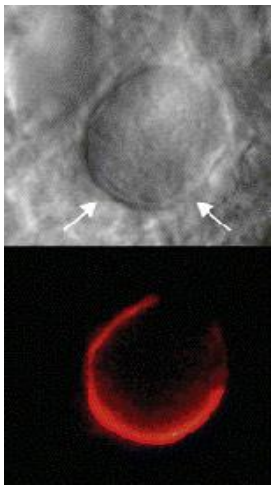

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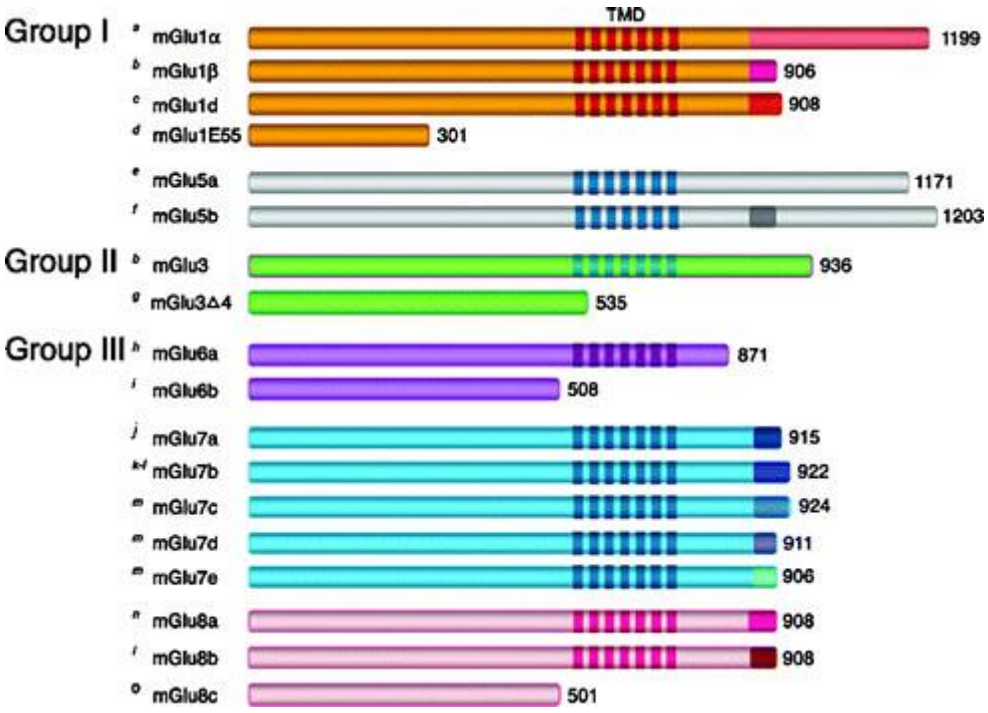

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- If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures,
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- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.
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Anexo 2. Comprovante de submissão do artigo.

Environmental Science and Pollution Research

Study of microbial degradation chloramphenicol by endophytic fungi isolated from Bertholletia excelsa (Brazil nuts)

--Manuscript Draft--

Manuscript Number:		
Full Title:	Study of microbial degradation chloramphenicol by endophytic fungi isolated from Bertholletia excelsa (Brazil nuts)	
Article Type:	Research Article	
Keywords:	Micropollutants; Brazilian nut; Antibiotic Biodegradation; Plant-microorganism; Phenicol antibiotics; Environmental toxicity.	
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	André L.M Porto	
	Irlon Maciel Ferreira, Ph.D	
Order of Authors Secondary Information:		
Funding Information:	FAPEAP (34568.515.22257.28052017)	Dr Irlon Maciel Ferreira
	FAPESP (2017/19721-0)	Dr Willian G. Birolli

Abstract:	Chloramphenicol (CAP), the compound aproached in this study, are a micropollutants and resists to conventional residual water treatment procedures. Thus, the biodegradation process employing specific and efficient microorganisms, including fungi, is an ecologically viable and low-cost option. Therefore, the aim of this study was to assess CAP biodegradability by five endophytic fungi strains isolated from Bertholletia excelsa collected in the Brazilian Amazonia. For this, the fungi strains were screened in solid/liquid medium and experimental design was performed to optimize culture conditions. In addition, an environmental toxicology assessment was carried out using the algae Chlorella vulgaris. Results from fungi cultures in solid medium demonstrated that CAP affected the strains growth and interfered in the development of conidia and spores. Moreover, the initial biodegradation screening showed that all strains managed to increase this antibiotic's degradation; Trichoderma sp. (BIORG 7), which was the strain that presented better results, was subjected to experimental design (Box-behnken) consisting of 15 experiments, having as variables:
	pH (5, 7, and 9), period (24, 48, and 72 hours), and CAP concentration (50, 100, and 150 mg.L-1), reaching a biodegradation yield (by HPLC-UV) of 30% (24h, pH 7,0 and 150 mg.L-1). The experimental design showed that the concentration has greater influence in the biodegradation process of the CAP by endophytic fungi. The metabolite 4-nitrobenzaldehyde was identified as a biodegradation product (by CG- MS) and product of biodegradation showed to be higher ecotoxicity in green algae. This metabolite that may be related with the diseases caused in different organisms.
Suggested Reviewers:	José Carlos Tavares Carvalho, Dr Universidade Federal do Amapa jctcarvalho@gmail.com
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