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

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ABSTRACT

Chloramphenicol (CAP) is a micropollutant that resists to conventional residual water treatment. 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. The fungi strains were screened in solid and liquid medium, and then 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. The biodegradation in liquid culture medium showed that all strains increased the degradation of this antibiotic. The most efficient strain *Trichoderma* sp. (BIORG 7) was subjected to an experimental design (*Box-behnken*) consisted of 15 experiments, having as variables: pH, time, and CAP concentration, and reaching 30% biodegradation determined by HPLC-UV analyses (24 h, pH 7.0 and 150 mg L^{-1}). The main metabolite 4-nitrobenzaldehyde was identified by GC-MS analyses and presented a higher ecotoxicity to green algae than CAP.

1. 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, mainly because these compounds can contribute 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). The antibiotic chloramphenicol (CAP), the target of this study, has been recurrently found in aquatic environments (Xiong et al., 2019).

CAP is a broad-spectrum antibiotic that has been commonly used to treat meningitis, plague, cholera, and typhoid fever, but it has limited use due to its carcinogenic, genotoxic and hepatotoxic effects in humans (Epaulard and Brion, 2010; 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 the nitro and chlorine groups present in this compound are responsible for the resistance to bacterial biodegradation, resulting in a persistence of CAP in 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) processes. However, there are

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few reports of aerobic biological processes (biodegradation) by fungus (Navada and Kulal, 2019), bacteria (Ma et al., 2019) or microalga species.

Biodegradation processes are environmentally friendly and represent a low cost option for micropollutants treatment (Alvarenga et al., 2014; Birolli et al., 2018). Generally, the employed microorganisms consume the 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).

The total biodegradation or mineralization involves the consumption of intermediate compounds, which can be more or less toxic substances than the starting compound (Serrano-González et al., 2018). These processes have several advantages, including low energy employment and green catalysis. Moreover, the biodegradation of different organic pollutants by fungi has been successfully employed (Alvarenga et al., 2014; Vacondio et al., 2015).

Endophytic fungi are promising biocatalysts that survive in the tissues of healthy plants without causing any infection in the host (Afzal et al., 2014). Additionally, studies have proven that these fungi can also be used in the degradation of organic compounds (Potin et al., 2004), i.e., phenanthrene by *Ceratobasidum stevensii* isolated from a *Euphorbiaceae* plant (Dai et al., 2010) and 4-hydroxybenzoic acid, ferulic acid, cinnamic acid and sinapic acid by *Phomopsis liquidambari,* isolated from *Bischofia polycarpam* (Chen et al., 2013; Fu et al., 2018).

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

2. Materials and methods

2.1. Reagents, solvents and culture media

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), isopropanol (HPLC grade) and acetonitrile (HPLC grade) were obtained from PANREAC and TEDIA, respectively.

2.2. Isolation and identification of endophytic fungi from Bertholletia excelsa

The seeds of *Bertholletia excelsa* (Brazil nuts) were collected and assigned by the 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 fungal endophytes used in this study were isolated from seeds and stored according to the protocol described by Ekiz et al., 2018.

The 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; Barnett and Hunter, 1998). For this biodegradation study, the five employed fungal strains were: *Aspergillus bertholletiae* BIORG 4, *Aspergillus* sp. BIORG 5, *Penicillium paxilli* BIORG 6, *Trichoderma* sp. BIORG 7, and *Aspergillus* sp. BIORG 9. The endophytic fungi (BIORG 4, BIORG 6 and BIORG 9) used in this work were identified by both conventional and molecular methods at the Chemical, Biological, and Agricultural Pluridisciplinary Research Center (CPQBA, https://cbmai.cpqba.unicamp.br) at the State University of Campinas (UNICAMP), SP, Brazil.

2.3. Growth of strains in the presence of the chloramphenicol

Endophytic fungi strains were cultured on solid culture medium containing Agar (2%) and Malt extract with pH adjusted to 7.0. Then the media was sterilized in an autoclave (Phoenix, AV50) at 121 °C for 20 min. After that, a solution of CAP (125 mg solubilized in 5 mL of DMSO) was supplemented to the culture media to the final concentration of 100 mg L^{-1} in the Petri dishes, it was noteworthy that the addition was performed at 40–50 °C to prevent thermal degradation (Vacondio et al., 2015). Subsequently, the medium was homogenized with gentle circular movements.

After 24 h, Petri dishes containing the Malt extract (2%) and Agar (2%) were inoculated with spores of each endophytic fungus using an inoculation needle through a central insertion point. Then, the fungi strains were incubated at 28 °C (BOD, LUCADEMA, model LUCA-161/03) by 9 days and monitored every 24 h by radial growth. Solid-medium plates without CAP were used as fungal control. All experiments on solid media were performed in duplicates.

2.4. Biodegradation of chloramphenicol in liquid medium

Five endophytic fungal strains were employed in an initial screening performed in 125 mL-Erlenmeyer flasks containing 50 mL of Malt liquid medium (2%) at pH 7.0. The inoculations were carried out with seven circular disks (0.5 cm of diameter) from a 7-days old solid culture. Each strain was incubated in an orbital shaker for 5 days (30 °C, 130 rpm) and then 100 mg L^{-1} of CAP (previously dissolved in 5 mL of DMSO) were added in the reaction medium. The biodegradation reactions were performed for 3, 6 and 9 days in an orbital shaker (30 °C, 130 rpm). The experiments were performed in triplicates.

To validate the developed method, five liquid culture media of 2% Malt were prepared in 125 mL-Erlenmeyer flasks containing 50 mL of culture medium. In each of them, seven circular disks (0.5 cm of diameter) of the fungal strain *Trichoderma* sp. BIORG 7 were inoculated, and the flasks were placed in an incubator for orbital shaking for 5 days, 30 °C and 130 rpm. Thereafter, the reactions were sterilized in autoclave for 20 min at 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.

It was also performed killed-cells controls, which were experiments to determine the contribution of the CAP adsorption in the cell walls, abiotic degradation and volatilization of the antibiotic for the same time of the biodegradation reactions (3 days). For this, three samples were prepared and sterilized as the experiments described for method validation. However, after sterilization and antibiotic addition, the inactived samples were placed in an orbital shaker for 76 h at 32 °C and pH of 6.6, which were the optimized conditions. Then, the samples were extracted and prepared for quantification by chromatographic analyses.

2.5. Biodegradation of chloramphenicol employing experimental design and statistical model

In this study, a three levels and three variables 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) in the initial screening of biodegradation. Reaction time (h), pH of the medium and CAP concentration (mg.L^{-1}), which presented significant effects in preliminary one-factor-at-a-time experiments were taken as the independent variables in a 15-run experiment to determine their optimum levels. The 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 was described in Equation (1):

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

Factor	Name	Levels		
		−1	0	+1
x1	Time (h)	24	48	72
x2	pH of the medium	5	7	9
x3	Chloramphenicol concentration (mg L^{-1})	50	100	150

$$Y = \beta 0 + \beta 1x1 + \beta 2x2 + \beta 3x3 + \beta 11 \times 1^2 + \beta 22 \times 2^2 + \beta 33 \times 3^2 + \beta 12 \times 1\text{-} \times 2 + \beta 13 \times 1 \times 3 + \beta 23 \times 2 \times 3 \quad (1)$$

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 the independent variables (Dong et al., 2009).

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 chart was produced for the obtainment of the significance of the tested variables on the mentioned responses.

2.6. Extraction of chloramphenicol and its metabolites

After the respective period of biodegradation, 50 mL of ethyl acetate was added to each reaction, resulting in an ethyl acetate and water (1:1) solution, which was maintained under vigorous magnetic stirring for 30 min. This process was used to promote the cells lysis, and after that the mixture was filtered. Then, the pH of the culture broth was measured and standardized to 7.0 (QUALXTRON). The aqueous phase was extracted with ethyl acetate (3 x 30 mL) and the organic phase was subsequently dried with anhydrous Na_2SO_4, filtered, and the excess of solvent was removed under reduce pressure in a rotevaporator. Then, the organic extract was solubilized in methanol (5 mL). The products were quantified by HPLC analyses and the metabolite identification was performed by GC-MS analysis using authentic standards.

2.7. Quantification of chloramphenicol by HPLC-UV analyses

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, UV-VIS SPD M20A detector, CTO-20A column oven and CBM-20A system controller. The separations were performed using a Phenomenex C18 Luna Column (5 µm of particle size, 25 cm × 4.6 mm). The material was eluted using a mixture of water (solvent A) and acetonitrile (solvent B), as follows: isocratic mode (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 an 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, concentrating them 10-fold. Therefore, standard solutions of 50, 350, 650, 950 and 1250 mg L^{-1} in methanol were employed for the acquisition of a standard curve for quantification of CAP, generating the linear equation: c = 26277.x + 187773.

2.8. Detection of metabolites by GC-MS analyses

The analyzes for the metabolites detection were performed by gas chromatography coupled to mass spectrometry in a Shimadzu/GC-2010 apparatus equipped with a 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 × 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 employed split ratio was 1: 1.

2.9. Environmental toxicology assay

Simultaneous environmental toxicity tests of the CAP and biodegradation products (crude extracts) were conducted using the green alga *Chlorella vulgaris*, as described by Oliveira et al. (2017) with some modifications. The toxicity tests were performed with a 10 mL of *C. vulgaris* inoculation in nitrogen, phosphorus and potassium (NPK, 08:08:08) aqueous solution. The initial cell density was 1×10^4 cells mL^{-1}. A separated aliquot of CAP (group 1), degradation products of CAP (group 2), fungal metabolites from crude extracts (group 3) in different concentrations 50, 100 and 150 mg mL^{-1} was added in the samples of *C. vulgaris*. A control group was made to compare the cell densities of the substrate.

The density of cells was quantified using a Neubauer chamber after 1, 3, 5, 10 and 15 days. Percentage of viable cells (%VC) was calculated as follows: %VC = (D/D0) × 100, where: D is the cell density at each specific day and D0 is the cell density before substrates addition. All the asssays were performed in triplicate.

3. Results and discussion

3.1. Fungal growth on solid medium

Radial growth tests with the strains isolated from Brazil nut tree containing CAP at 100 mg L^{-1} or only Malt 2% medium as control experiment 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 h. The results are shown in Table 2.

For the strain *Aspergillus bertholletiae* BIORG 4, the presence of CAP induced a slight increase of the colony diameter, when compared to the control experiment containing only Malt 2%. Suggesting that this fungus may use the antibiotic as source of carbon, hence inducing mycelial growth. While the strain reached 8.0 cm of 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 paxilli* 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 the 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).

For all the available endophytic fungi (*Aspergillus bertholletiae* BIORG 4, *Aspergillus* sp. BIORG 5, *Penicillium paxilli* BIORG 6, *Trichoderma* sp. BIORG 7, and *Aspergillus* sp. BIORG 9) incubated on solid medium in the presence of CAP was observed a conidiogenesis

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

Strains	Growth medium	Time (days)		
		3	6	9
		Colony diameter[a] (cm)		
Aspergillus bertholletiae 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 paxilli 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 diameters 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 2%) and 22 days for the control experiment (Malt 2% + CAP).
[c] The diameter of the colony strain was performed every 24 h due to its fast growth.
[d] The colonies (Malt 2% and Malt 2% + CAP) reached the maximum diameter of 8.0 cm after 11 days of growth. CAP: Cloramphenicol.

Table 3
Mycelial growth of *Trichoderma* sp. BIORG 7 on solid medium with central insertion point (100 mg.L^{-1} of CAP per plate).

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

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

process, and a consequently decrease in the production and retardation of spore maturation, but with no effect on growth. The necessary conditions for sporulation, as well as secondary metabolism, are generally more specific than the conditions for vegetative growth (Sekiguchi and Gaucher, 1977).

Similarly, it was observed toxic effects of Imidacloprid and Fipronil on *Beauveria bassiana* and *Metarhizium anisopliae* fungi, causing a decrease in conidia production (Moino and Alves, 1998). The interference in the conidia production and fungal growth by the presence of pesticides and fungicides (thiophanate methyl, cartape, methyl parathion, tebuconazole and tetraconazole) in strains of entomopathogenic fungi was also reported (Loureiro, 2012).

This inhibition could be a result of a nocive effect caused by the antibiotic, or due to some metabolite formed during the CAP biotransformation. Further experiments revealed that this inhibition was due to the production of 4-nitrobenzaldehyde or other biodegradation product, which is toxic for microorganisms in general. The compound 4-nitrobenzaldehyde is a thermally stable substance employed as intermediate in organic synthesis for the production of dyes and biologically active products. Besides that 4-nitrobenzaldehyde is not efficient in the block of singlet oxygen generation under aerobic conditions, facilitating oxidation of lipids and proteins (Hajimohammadi et al., 2018). Other investigations about the degradation of CAP, such as, radiation degradation (Hong et al., 2002), photodegradation (Lofrano et al., 2016) and microbial degradation (Ma et al., 2019) also identified 4-nitrobenzaldehyde as a degradation product.

3.2. 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 demonstrated the precision of the developed method. The proposed method may show great applicability for other substrates in biodegradation studies.

3.3. 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, the culture in liquid medium was performed to evaluate each fungi efficiency in the biodegradation of this antibiotic.

These strains were cultured for 5 days in Malt 2% liquid medium in orbital stirring containing 100 mg L^{-1} of CAP. Biodegradation reactions were performed for 3, 6 and 9 days. These results indicated that all tested microorganisms 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 strains *Trichoderma* sp. BIORG 7 and *Aspergillus* sp. BIORG 9 (25.2% and 29.3%, respectively). Based on the results of average biodegradation and standard deviation, the strain *Trichoderma* sp. BIORG 7 was selected for further experiments in different periods of biodegradation, pH and concentration of CAP for optimization of the biodegradation process.

3.4. Evaluation of chloramphenicol biodegradation through experimental design

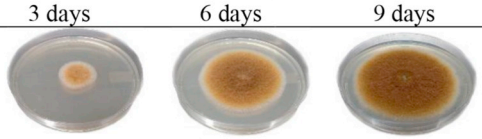
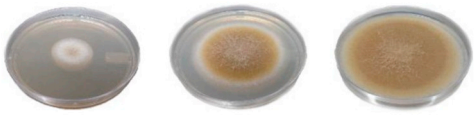
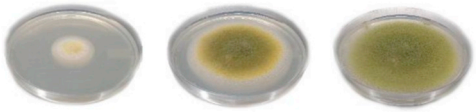
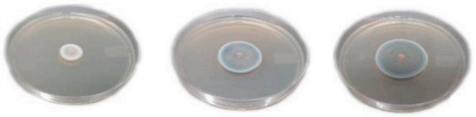
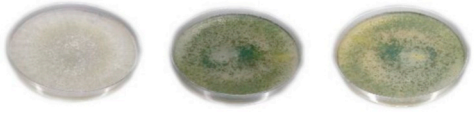
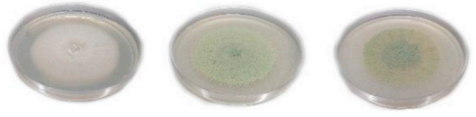
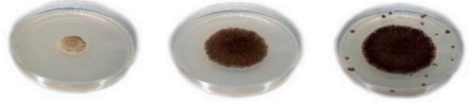
Based on the best results of the previous section, CAP biodegradation content (BD%) was obtained for different trials of the experimental design protocol with the fungus *Trichoderma* sp. BIORG 7. Variables values are showed in Table 6 and a Pareto chart of standardized effects presented in Fig. 1 showed significant effects of CAP concentration, reaction time and pH of the medium variables (quadratic). The bar length of each parameter characterizes the absolute importance of the estimated effect. The vertical line represents the limit between the significant and non-significant 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*) was presented in Fig. 2. Interactions between these two factors showed that the reaction time does not interfered on the response for the evaluated periods.

On the other hand, Fig. 2 showed that the 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 for pH 7.0 at the highest CAP concentration (150 mg L^{-1}). For Xin et al. (2012), the medium pH of 7.0 was also determined for maximal CAP degradation rates, where aerobic strains of intestinal bacteria (*Klebsiella pneumonia* and *Escherichia fergusonii*) were employed in reactions of 7 days.

In some cases, the filamentous fungi can have advantages in the biodegradation of organic pollutants, because it has greater cell

Table 4
Microbial growth experiments for fungi strains isolated from Brazil nut for CAP biodegradation on solid medium with central insertion point (100 mgL^{-1} of CAP per plate).

Strain	Growing medium	Colony Growth		
		3 days	6 days	9 days
Aspergillus bertholletiae BIORG 4	Malt 2%			
	Malt 2% + CAP			
Aspergillus sp. BIORG 4	Malt 2%			
	Malt 2% + CAP			
Penicillium paxilli BIORG 6	Malt 2%			
	Malt 2% + CAP			
Trichoderma sp. BIORG 7	Malt 2%			
	Malt 2% + CAP			
Aspergillus sp. BIORG 9	Malt 2%			
	Malt 2% + CAP			

stability compared to prokaryotes (Olicón-Hernández et al., 2017). However the biodegradation of CAP was recently studied by Ma et al. (2019) and showed that an efficient consortium of bacteria (*Sphingobium* sp., *Pandoraea* sp., *Comamonas* sp., *Pseudomonas* sp. and *Cupriavidus* sp.) can be very efficient in the degradation of CAP, reaching 63% of biodegradation in 24 h.

CAP concentration (*x3*) and reaction time (*x1*) were statistically the most significant factors. According to the results presented in Fig. 3, BD % increased when CAP concentration (*x3)* was increased from 50 to 150 mg L^{-1} for 24 h (Table 6). However, good results were also observed at 48 h, although the most significant biodegradation rate was in the shortest experimental time (24 h) with the highest concentrations of antibiotic. Fig. S3 (*Supplementary Information*) provides the chromatogram obtained by HPLC-UV through the experimental design.

3.5. Identification of 4-nitrobenzaldehyde as metabolite from chloramphenicol biodegradation

The biodegradation extracts were analyzed by GC-MS for metabolites detection and identification. The retention time of the intermediate

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	85.9 ± 3.3
	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	70.7 ± 20.1
CAP biodegradation (%)	3	8.5	21.1	9.1	23.3	14.1
	6	24.2	26.9	26.5	24	22.4
	9	25.3	24.6	23.1	25.2	29.3

[a] Abiotic control: 97.9 ± 1.1.

Table 6
The design matrix and responses for the independent variables levels available in CAP biodegradation by *Trichoderma* sp.

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

from CAP degradation and 4-nitrobenzaldehyde standard were the same at 19.3 min. Resulting in a common spectra with *m/z* 150 as base peak, and fragmentation ions of *m/z* 120, 105 and 97 (*Supplementary Information*). A similar result was reported for Ma et al. (2019), when 4-nitrobenzaldehyde was identified as an intermediate product of CAP biodegradation by *Sphingobium*.

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.

According to Xin et al. (2012), the biodegradation of CAP by *Klebsiella pneumonia*, follows initially a hydrolysis into 1-*p*-nitropheny-2-amino-1,3-propanediol as well as dichloroacetamide, and then a convertion into CO_2 + H_2O + NH_4^+ + Cl^-, or a hydrolysis of the amide bond to produce *p*-nitrophenylserinol, which could be further transformed to a small amount of *p*-nitrobenzaldehyde, *p*-nitrobenzyl alcohol, and *p*-nitrobenzoic acid (Malik and Vining, 1970, 1971; Mosher et al., 1990).

Chloramphenicol has been associated with aplastic anemia in humans and, reproductive and 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 mechanism of disease cause in eukaryotic cells.

3.6. Environmental toxicology assays

In the experiments containing the biodegradation products of CAP (Fig. 3A), a slight decrease of cells number compared to the initial amount was observed in the first 24 h, especially in the samples with concentrations of 100 and 150 mg L^{-1} (0.73 and 0.72 × 10^6 cell mL^{-1}, respectively). After that, the number of *Chlorella vulgaris* cells remained linear in the other determinations with variations of 0.55 × 10^6 to 1 × 10^6 in the three concentrations until the tenth day of the experiment. In the last cell counting occurred a significant decline of population in all concentrations – 0.15 × 10^6 (50 mg L^{-1}), 0.25 × 10^6

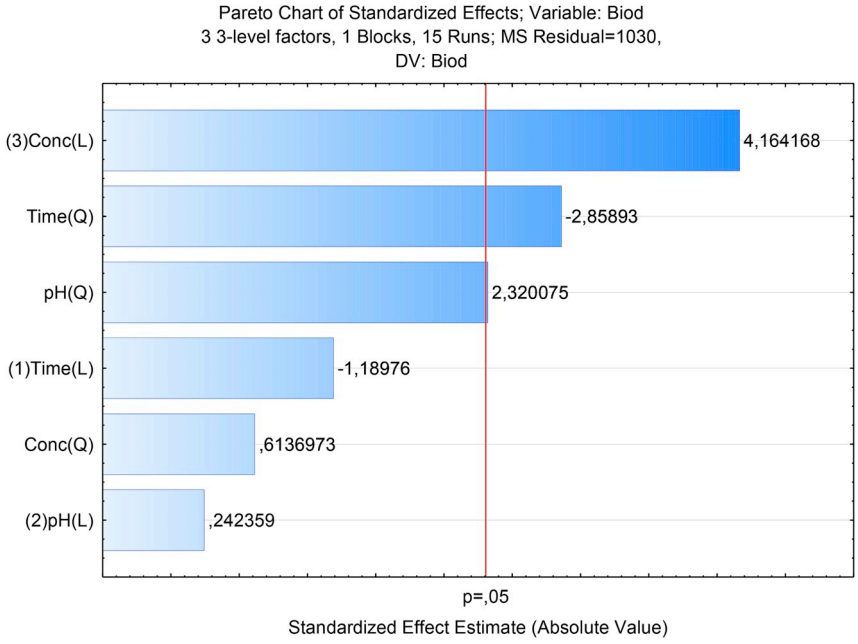


Fig. 1. Pareto chart illustrating main and interaction effects of the factors affecting the CAP biodegradation content (%); (1) Time (L): linear effect of factor *x1*, (2) pH (L): linear effect of factor *x2*, (3) Conc (L): linear effect of factor *x3*, Time (Q): quadratic effect of factor *x1*, pH(Q): quadratic effect of factor *x2*, Conc (Q): quadratic effect of factor *x1*. The vertical line represents the limit between the significant and non-significant effects with a 5% risk of error.

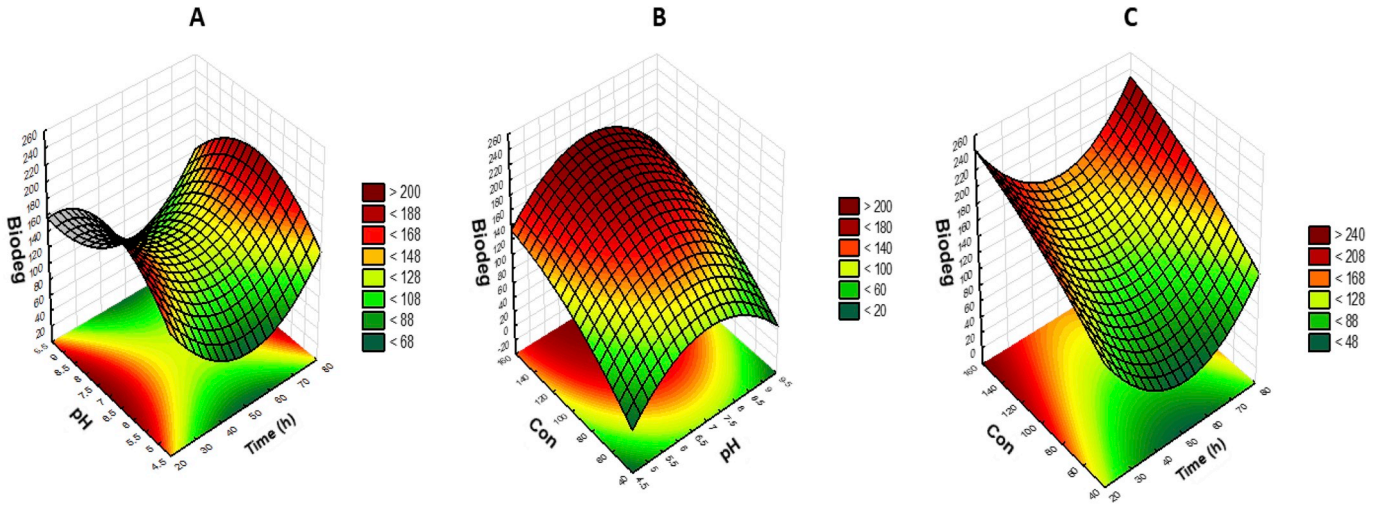


Fig. 2. Response surface plot and contour plot of the CAP biodegradation content as a function of (A) reaction time (*x1*) and pH of the medium (*x2*); (B) pH of the medium (*x2*) and CAP concentration (*x3*); (C) CAP concentration (*x3*) and contact time (*x1*).

(100 mg L^{-1}), and 0.3×10^6 (150 mg L^{-1}) cell mL^{-1}, indicating a toxic effect after the tenth day of experiment on the algae caused by the metabolites produced during the CAP biodegradation (Fig. 3A), when compared to the control experiment.

Regarding the experiments performed only with the metabolites produced (crude extract) by the fermentation of the fungi *Trichoderma* sp. (without CAP), there was a decrease in the concentration of cells in relation to the control group. The algal cell density rates regressed discretely during the experiment (Fig. 3B). On the last day of counting, the values were 0.33 for 50 mg L^{-1}, 0.30 mg L^{-1} for 50 mg L^{-1}, and 0.33×10^6 for 150 mg L^{-}, indicating that the crude extract from the fermentation of *Trichoderma* sp. in liquid mediumaffected the growth rate of algae in the employed conditions.

In the experiments with CAP solution alone (Fig. 3C), the results showed pronounced decrease of cell quantity in all concentrations, i.e., from 0.68×10^6 to 0.4×10^6 (50 mg L^{-1}), from 0.48×10^6 to 0.35×10^6 (100 mg L^{-1}), and from 0.6×10^6 to 0.35×10^6 cell mL^{-1} (150 mg L^{-1}). Overall, the solution of CAP led to a decreased 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*.

Recent studies have shown that the toxics effects of CAP in some species of algae (*Pseudokirchneriella subcapitata*, *Scenedesmus quadricauda*, *Scenedesmus obliquus* and *Scenedesmus acuminatus*) promoted negative effects on the growth and alteration of biochemical components, as the composition and structure of lipids, proteins and DNA (Xiong et al., 2019).

4. Conclusion

This was the first study that showed the use of endophytic fungi in the biodegradation of the micropollutant CAP. The strains *Aspergillus* sp. BIORG 9 and *Trichoderma* sp. BIORG 7 presented the best results of biodegradation, 29.3% and 25.2% of biodegradation for 9 days, respectively. The experimental design applied to the strain *Trichoderma* sp. BIORG 7 was essential for the optimization of the experimental conditions. Reducing the employed resources as proposed by the green chemistry principles, since the maximum experimental period was 72 h, 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, followed by the reaction time in second. In brief, the best biodegradation conditions with the selected microorganism was 24 h, pH 7, and CAP concentration of 150 mg L^{-1}.

This study showed that endophytic fungi, including *Trichoderma* sp. BIORG 7, showed potential as biocatalysts for future green processes and can be able to improve the biodegradation of other contaminants, including in different approaches as the employment of microbial

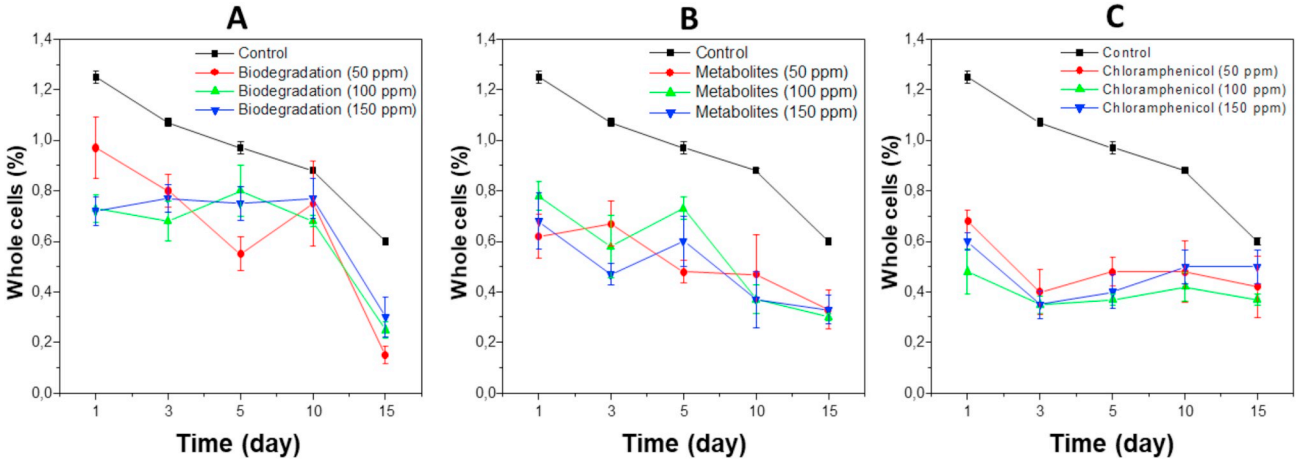


Fig. 3. *Chlorella vulgaris* number of cells for toxicity evaluation (A) CAP biodegradation products; (B) Crude extract from fungal metabolites; (C) Chloramphenicol solution.

consortia and other associations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101200.

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