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Influence of the carbon-to-nitrogen ratio in the atrazine biodegradation from contaminated waters in submerged fermentation by free and immobilized *Penicillium chrysogenum* NRRL 807

Influência da relação carbono-nitrogênio na biodegradação de atrazina em águas contaminadas por fermentação submersa com Penicillium chrysogenum NRRL 807 na forma livre e imobilizada

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ABSTRACT

Atrazine is a pesticide commonly used in agriculture and is recognized as a potent endocrine disruptor. Due to its high recalcitrance, its residues have been found in drinking water sources throughout Brazil and the world. Therefore, this study aimed to evaluate the influence of the C/N ratio on the potential of the fungus *Penicillium chrysogenum* NRRL 807 to degrade atrazine from contaminated waters in submerged fermentation. Moreover, the free and immobilized forms of the fungus were compared. The fungus grown in suspended culture (free form) was able to degrade 40.08±5.71% of the atrazine present in the medium after 5 days, while the immobilized form (biofilm) degraded 48.31±1.53% in the same incubation time. Notably, atrazine was used as a carbon source, and degradation was led by the enzyme complex of the cytochrome P450. The amount of exogenous nitrogen was determined to interfere with the biodegradation efficiency, diverting the metabolism to the path of spore germination when nitrogen was present in high concentrations. Based on these results, *P. chrysogenum* both in its free form and when immobilized in biofilms can be used as bioremediation technologies for treating water contaminated by atrazine.

Keywords: pesticides; fungus degradation; exogenous nitrogen; biofilms; bioremediation technology.

RESUMO

A atrazina é um pesticida comumente utilizado na agricultura, reconhecido como um potente disruptor endócrino. Dada a sua elevada recalcitrância, seus resíduos têm sido encontrados em mananciais de água potável pelo Brasil e pelo mundo. Diante disso, este estudo teve como objetivo avaliar a influência da relação C/N no potencial do fungo *Penicillium chrysogenum* NRRL 807 para degradar a atrazina em águas contaminadas em fermentação submersa, tanto em sua forma livre como na imobilizada (biofilmes). O fungo cultivado em cultura livre foi capaz de degradar 40,08±5,71% da atrazina presente no meio após cinco dias, enquanto o fungo imobilizado degradou 48,31±1,53% no mesmo tempo de incubação. Notavelmente, a atrazina foi utilizada como fonte de carbono e a degradação foi liderada pelo complexo enzimático do citocromo P450. Determinou-se que a quantidade de nitrogênio exógeno interfere na eficiência da biodegradação, desviando o metabolismo para a via de germinação de esporos quando o nitrogênio estava presente em altas concentrações. Com base nesses resultados, *P. chrysogenum* tanto em sua forma livre como em sua forma imobilizada em biofilmes pode ser usado como tecnologia de biorremediação para o tratamento de águas contaminadas por atrazina.

Palavras-chave: pesticidas; degradadação por fungos; nitrogênio exógeno; biofilmes; tecnologia de biorremediação.

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Introduction

Despite the risks it can pose to the environment and human health, the herbicide atrazine has been used globally in agriculture, and due to its high recalcitrance, its residues have been found in the environment (Bachetti et al., 2021; Urseler et al., 2022). The risks imposed by the strong endocrine-disrupting potential of atrazine include changes in the male development of amphibians, fish, reptiles, and mammals, as well as alterations in human cells (Blahova et al., 2020; Sánchez et al., 2020; Galbiati et al., 2021). Despite the high stability of the atrazine molecule and its resistance to microbial degradation, bioremediation techniques have been used to decontaminate environments, especially because they are more cost-effective and environmentally friendly (Mili et al., 2023).

Microbial bioaugmentation consists of adding some microorganisms capable of degrading a specific pollutant in a contaminated environment to degrade the substance more rapidly. The survival of these microorganisms will depend on a plethora of factors, including the suitability of the environment, the predation and competition of indigenous microorganisms, and the percolation of the bioaugmented microorganisms in the leachate.

Several fungi are able to remove the atrazine from contaminated environments through three mechanisms of action: biodegradation by the action of extracellular enzymes, such as the laccases; intracellular biodegradation, through the cytochrome P450 enzyme; and biosorption by the fungal biomass (Henn et al., 2020; Lopes et al., 2020; Lu et al., 2021). One of the first studies that investigated the degradation of atrazine by fungi was performed by Kaufman and Blake (1970), in which several species were able to degrade atrazine by N-dealkylation. Since then, several studies have reported the ability of soil fungi, such as some species of the genera *Aspergillus*, *Bjerkandera,* and *Penicillium,* in the degradation of atrazine (Yu et al., 2018; Dhiman et al., 2020; Herrera-Galardo et al., 2021).

The *Penicillium* fungi are widely distributed decomposers in soils, known to tolerate adverse environmental conditions. They have the P450 enzymatic complex, which allows the degradation of organic matter by dealkylation and dehalogenation, entitling them to applications in biodegradation and bioremediation (Zhang et al., 2021). Furthermore, *Penicillium chrysogenum* is also known to produce laccases (Eldin et al., 2022). Both systems, i.e., laccase production and P450 cytochrome, may act synergically on the bioremediation of atrazine-contaminated sites.

Some fungi also perform well in their free form (Yu et al., 2018), but the immobilization of the cells creates a more suitable environment with lower stress levels and provides higher concentrations of biomass (Mishra et al., 2022). Moreover, studies have shown interesting results with immobilized fungi (Tang et al., 2022). The study of both forms is, therefore, important because it contributes to the expansion of technologies that can be used in specific bioremediation projects for water or soil.

In this context, this study evaluated the potential for biodegradation of atrazine by the fungus *P. chrysogenum* NRRL 807 in its free and immobilized form. The influence of the C/N ratio was also evaluated. In this way, the investigated biotechnological processes may subsidize bioremediation techniques.

Materials and Methods

Microorganism

P. chrysogenum NRRL 807 was obtained from the collection of cultures of the Agricultural Research Service (ARS), Illinois, USA. The spores were kept in sterile sand at –80°C in an ultra-freezer (JJ Cientifica 200-80). To carry out the experiments, the spores were activated in Potato Dextrose Agar (PDA) for 5 days at 30°C and stored in a plate at 4°C for up to 2 months.

Chemicals and media

Atrazine and hydroxyatrazine (99% and 98.8% purity, respectively) were acquired from Sigma-Aldrich Brazil Ltda. The other chemical compounds used in this study were analytical grade, and all the reagents used in high-performance liquid chromatography (HPLC) analyses were of HPLC grade. The minimum mineral medium (MMM) used in the assays was composed of 0.25 g/L KH_2PO_4, 0.125 g/L KCl, 0.25 g/L $MgSO_4{\cdot}7H_2O$, and 0.0025 g/L $FeSO_4$ (Yu et al., 2018). Atrazine was solubilized in the medium with 4% methanol (v/v). To prepare the inoculum for immobilized fungi, the Czapeq's medium, composed of 1.0 g/L KH_2PO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.01 g/L $FeSO_4$, 3.0 g/L $NaNO_3$, and 30.0 g/L glucose, was used. The pH was adjusted to 7.0–7.2 before autoclaving at 121°C for 20 min. At the end of the tests, the pH was checked to verify its stability.

Atrazine biodegradation by free *P. chrysogenum* NRRL 807

The spores were removed from the PDA plates after 5 days of incubation at 30°C (Tecnal, TE-392/I), with a 0.5% Tween solution (v/v), and were released in a submerged fermentation system. An inoculum concentration of 1.0×10^6 spores was used in a reactional volume of 100 mL (in MMM). The experiments were carried out for 5 days, in triplicate, at 3.15×*g*, 30°C (rotary incubator Tecnal TE-421), and pH 7.0.

Influence of the carbon and nitrogen sources on atrazine degradation by free *P. chrysogenum* NRRL 807

The effect of the nutrient source on the degradation of atrazine was evaluated as fungi can use atrazine as both a carbon and a nitrogen source. Thus, the MMM containing atrazine was supplemented with exogenous sources of carbon and nitrogen at concentrations of 1.0 g/L of glucose and 0.75 g/L of $NaNO_3$, respectively. Four conditions were

compared: (a) MMM+glucose+$NaNO_3$ (ATZ+CN); (b) MMM+glucose (ATZ+C); (c) MMM+$NaNO_3$ (ATZ+N); and MMM, only (ATZ). These treatments were based on the study of Yu et al. (2018). In the first moment, only the effect of the nutrient source on the atrazine degradation by free *P. chrysogenum* at the standard initial concentration of 25 mg/L during 5 days was evaluated. In the group with the best biodegradation performance verified, two other initial concentrations of atrazine were analyzed: 10 and 50 mg/L. The influence of the exogenous nitrogen source concentration ($NaNO_3$) on the atrazine degradation by free *P. chrysogenum* NRRL 807 was also investigated using the initial atrazine concentration that demonstrated the best performance.

The exogenous sources concentrations tested were 0.05 g/L $NaNO_3$ (equivalent to 36 mg/L NO_3^-, C/N ratio 1.3); 0.5 g/L (365 mg/L NO_3^-, C/N ratio 0.7); 0.75 g/L (547 mg/L NO_3^-, C/N ratio 0.5); and 1.0 g/L (730 mg/L NO_3^-, C/N ratio 0.4). A treatment was also carried out without adding any exogenous source, with only atrazine (C/N ratio 1.4). The C/N ratio was determined considering the molecular weight of nitrogen and carbon present in atrazine and in the ATZ+N medium components. The percentage of degradation was calculated by taking into account the difference between the initial and final concentration of atrazine during the experiments.

Atrazine biodegradation by immobilized *P. chrysogenum* NRRL 807

The influence of the exogenous nitrogen source concentration ($NaNO_3$) on the atrazine degradation was also investigated using immobilized *P. chrysogenum* NRRL 807. The spores were removed (1.0×10^8 spores/mL) from the PDA plates after 5 days of incubation at 30°C with 0.5% Tween 80 solution (v/v). A volume of 10 mL of this solution was added to 250 mL — Erlenmeyer flasks containing 90 mL of Czapeq's medium (10% v/v), which also contained the inert support composed of three polyester meshes sewn together of about 5 cm^2 and with the pore width varying between 0.5 and 1 mm. The immobilized culture was formed under conditions of low agitation (0.9×*g*) for 5 days. Then, it was gently transferred with the support to the degradation tests Erlenmeyers using a tweezer, under sterile conditions. The other parameters of the degradation tests were the same as those used in the evaluation with free *P. chrysogenum* NRRL 807. The conditions for the formation of the fungal biofilm were based on the study by Šlosarčíková et al. (2017). All experiments were carried out in triplicate.

Determination of atrazine and hydroxyatrazine

Residual concentrations of atrazine and hydroxyatrazine were quantified by HPLC (Shimadzu Co., Japan) equipped with UV detector SPD-10AVVP at 209 nm and column Shim-CLC-ODS C18 (4.6×150 mm, 5 µm). The mobile phase consisted of methanol/phosphoric acid buffer pH 2.8 (70/30 v/v) with a flow rate of 1.0 mL/min, and the column temperature was 35°C. The samples were filtered in a membrane of 0.22 µm before each injection. The injection volume was 20 µL. The calibration curves and their respective adjustment values were determined before the experiments.

Biomass and sporulation evaluation

The biomass evaluation in the tests performed with the free *P. chrysogenum* was analyzed qualitatively through photographic records at the end of the experiments. Regarding immobilized biomass, in turn, it was determined gravimetrically (Šlosarčíková et al., 2017). The immobilized biofilms were gently removed from the Erlenmeyers using a tweezer, washed with distilled water, and dried at 70°C for 24 h. Some supports with immobilized biomass were separated at the beginning of the experiments to be weighed, totaling nine replicates, to evaluate the initial dry mass. In addition, polyester supports were also weighed to discount their dry mass.

The sporulation that occurred in the tests with immobilized biomass was analyzed in terms of optical density (DO_{600}) using a spectrophotometer (Genesys 10 uv, Thermo Scientific) on the third and fifth test days. The sample was analyzed under an optical microscope (Olympus BX51, Japan) to confirm the presence of spores and to rule out the possibility of bacterial contamination.

Enzymatic assays

The laccase activity was determined by spectrophotometry (Genesys 10 uv, Thermo Scientific) at 420 nm using 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) — ABTS as substrate (Valle et al., 2014). The manganese-peroxidase (MnP) activity was determined at 610 nm using phenol red as substrate (Khindaria et al., 1994). The activities were defined as the amount of enzyme capable of oxidizing 1.0 µmol of substrate per minute (U/L), under the conditions of the tests, taking into account the molar extinction coefficient (ε) for each substrate (ABTS, 36000 $M^{-1}cm^{-1}$; phenol red, 22000 $M^{-1}cm^{-1}$).

Statistical analysis

The results are presented as means with their standard deviations for each treatment. To determine the difference between the means in the treatments, single-factor variance analysis (one-way ANOVA) and Tukey test were performed, with a significance of $p<0.05$.

Results and Discussion

Nutrient source effect and carbon-to-nitrogen ratio influence on atrazine biodegradation by free *P. chrysogenum* NRRL 807

First, the effect of the nutrient source on the degradation of atrazine by the free *P. chrysogenum* NRRL 807 cultivated in submerged fermentation was evaluated. Out of the four conditions analyzed at the standard initial concentration (i.e., 25 mg/L of atrazine), some degradation was observed only in the ATZ+N, ATZ+C, and ATZ conditions after

5 days. However, only the ATZ+N condition yielded significant degradation relative to the initial concentration, 21.52±0.79% (Figure 1). The degradation in the ATZ+N medium was subtle and occurred slowly and steadily. This result indicates that the atrazine, under the conditions tested, served as a carbon source for the microorganism, as no other source of this element was added besides the pesticide itself. Furthermore, only the nitrogen present in the atrazine molecule may have been insufficient to promote rapid germination of the spores, resulting in a lower rate of atrazine degradation by the fungus compared with the addition of nitrate to the medium. Yu et al. (2018) also found greater degradation efficiency when atrazine was available as a carbon source for a spore solution of *Penicillium* sp. yz11-22N2 encapsulated. Glucose is the most easily assimilated substrate; therefore, when it is present in the environment, the genes that are used to metabolize other carbon sources end up being repressed to save energy (Marinho et al., 2017). Wang et al. (2021) also observed that fungi exhibit a broad spectrum of substrate utilization strategies, with genera from *Ascomycota* demonstrating dominant responsiveness to labile carbon utilization.

Thus, as the only significant degradation was obtained in the ATZ+N treatment, the effect of other initial standard concentrations of atrazine on this condition was investigated (Figure 2). At 10 mg/L, there was no significant degradation relative to the initial concentration. However, when using 50 mg/L, a degradation efficiency of 11.06±2.95% was observed. The biodegradation efficiency yielded at 25 mg/L (21.52±0.79%) can be justified by the greater amount of carbon, present in the atrazine molecule, which increased as the initial concentration also increased. This pattern is quite common to be observed, having been described in several studies (Zhao et al., 2017; Yang et al., 2018; Wirsching et al., 2021). It is noteworthy that, when using 25 mg/L, the fact that atrazine served as a carbon source was more prevalent than its toxicity to the free *P. chrysogenum*, even though the degradation was subtle. On the contrary, the lower degradation observed at 50 mg/L also points to the influence of its toxicity to the microorganism at higher concentrations. This pattern was corroborated by Farhan et al. (2021). These results indicate that there must be an optimal intermediate concentration for the degradation to occur.

Although the degradation, in percentage, was greater at the initial concentration of 25 mg/L compared with that of 50 mg/L, the amount of atrazine consumed in both concentrations during the test was the same (ca. 5 mg/L in 5 days; Figure 2). This implies that the rate of degradation was also the same in both situations. It can be inferred from this that the maximum capacity of free *P. chrysogenum* NRRL 807 to degrade atrazine under the conditions tested may have been achieved, perhaps due to the lack of some other important nutrients, for example.

After it was found that the degradation of atrazine occurred more significantly at the initial concentration of 25 mg/L and with the addition of an exogenous source of nitrogen ($NaNO_3$), the concentration of this source was varied in tests using free and immobilized *P. chrysogenum* NRRL 807 (Figure 3). In the tests with the free form, the highest degradation was observed with the addition of 0.05 g/L of the exogenous nitrogen source, i.e., the lowest concentration tested. In this case, an average value of 40.08±5.71% was yielded, which represents an increase of about 18.5% in the degradation efficiency compared with the efficiency performed by the free form at the standard concentration of 0.75 g/L $NaNO_3$ (Figure 2). When analyzing the influence of the exogenous nitrogen source on the degradation of atrazine by spores of *Penicillium* sp. yz11-22N2 encapsulated, Yu et al. (2018) also found that excessive concentrations did not improve the degradation efficiency.

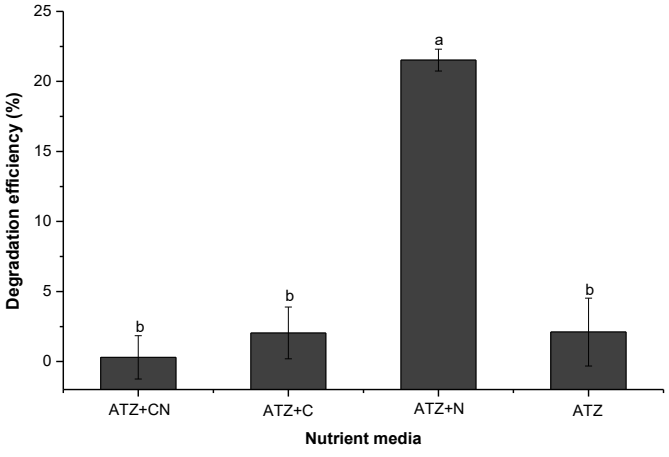

Figure 1 – Atrazine degradation efficiency by free P. *chrysogenum* NRRL 807 in submerged fermentation after 5 days in minimal mineral medium with different nutrient substances (ATZ+CN, medium with 25 mg/L of atrazine supplemented with 1.0 g/L glucose and 0.75g $NaNO_3$; ATZ+C, atrazine and glucose; ATZ+N, atrazine and $NaNO_3$; and ATZ, atrazine only). Error bars correspond to the mean values±standard deviation. Different letters indicate statistical differences (p<0.05).

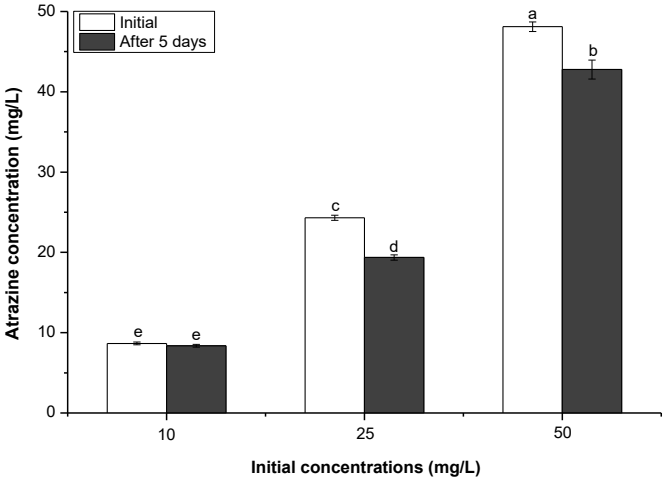

Figure 2 – Atrazine concentration (mg/L) at the beginning of the test and after degradation by free P. *chrysogenum* NRRL 807 for 5 days in the minimal mineral medium at the initial concentrations of 10, 25, and 50 mg/L of atrazine with nitrogen as exogenous source (0.75 g/L $NaNO_3$). Error bars correspond to the mean values±standard deviation. Different letters in each period indicate statistical differences (p<0.05).

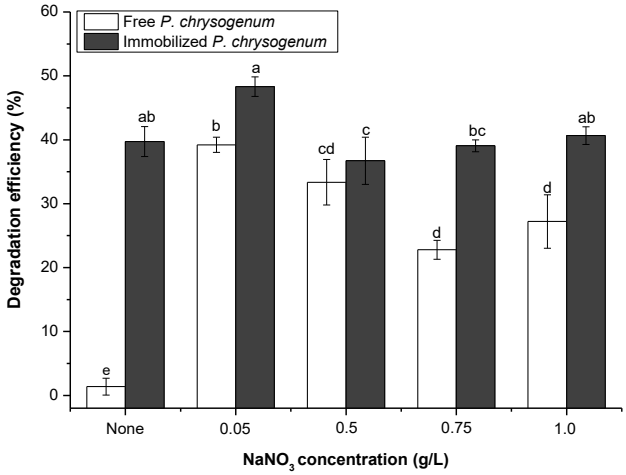

Figure 3 – Atrazine degradation efficiency by free and immobilized P. *chrysogenum* NRRL 807 after 5 days in minimal mineral medium with atrazine initial concentration of 25 mg/L in different concentrations of an exogenous source of nitrogen ($NaNO_3$). Error bars correspond to the mean values±standard deviation. Different letters in each period indicate statistical differences ($p<0.05$).

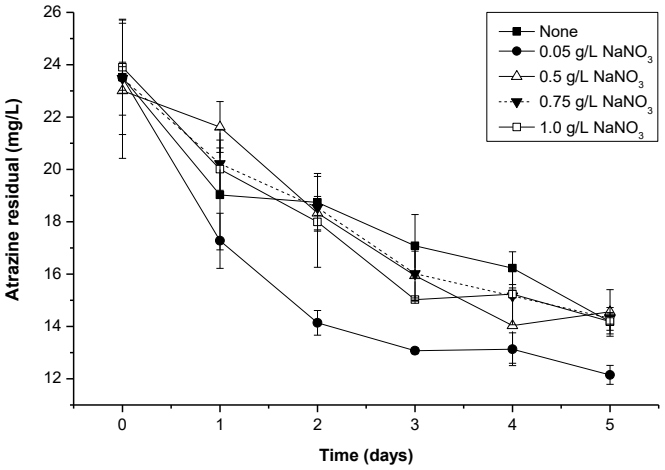

Figure 4 – Atrazine residual concentration (mg/L) during degradation in 5 days by immobilized P. *chrysogenum* NRRL 807 immobilized in minimal mineral medium with atrazine initial concentration of 25 mg/L in different concentrations of exogenous nitrogen source ($NaNO_3$). Error bars correspond to the mean values±standard deviation.

Nutrient source effect and carbon-to-nitrogen ratio influence on atrazine biodegradation by immobilized *P. chrysogenum* NRRL 807

The performance profile observed in the tests with free *P. chrysogenum* NRRL 807 was also confirmed with its immobilized form. The best performance was obtained when the exogenous nitrogen source concentration was 0.05 g/L, 48.31±1.53% (Figure 4).

In general, immobilized *P. chrysogenum* NRRL 807 was able to degrade more atrazine than the free form. In addition, the fungus was able to degrade atrazine in a mineral medium without the supplement of any other exogenous nutrient source besides the pesticide itself (39.73±2.34%), unlike the free form, for which no significant degradation was observed. The biofilm of *P. chrysogenum* NRRL 807 was transferred to the ATZ+N medium after its formation in Czapeq's medium. It is possible, therefore, that the fungus converted the nitrogen already present in its biomass to use in the metabolic degradation pathway. In any case, the higher C/N ratio present in the medium, due to the lower amount of nitrogen added, seemed to promote the degradation of atrazine. Thus, in conditions where there is a higher nitrogen supply (lower C/N ratio), the metabolic route was deviated, favoring the degradation.

The improvement in the performance of the immobilized *P. chrysogenum* NRRL 807 in the degradation of atrazine can be attributed to the greater amount of inoculum. Moreover, the inoculum was added when the microorganism was in an active growth phase. One possibility that must be taken into account is the ability of the mycelium to adsorb atrazine. Many studies have reported the removal of pesticides, metal ions, textile industry dyes, and organic pollutants by biosorption by fungi or mycelial pellets (Cabrera-Barjas et al., 2020; Cheng et al., 2020; Nouri et al., 2021).

The chromatograms from the analysis of the residual atrazine at the end of the tests reinforce the hypothesis of the atrazine degradation being performed intracellularly by both free and immobilized *P. chrysogenum* NRRL 807 through the action of the cytochrome P450 enzymatic system. In these chromatograms, it is possible to identify peaks that can be considered metabolites of the atrazine degradation (Figures 5A and 5B) which were not present in the chromatograms at the beginning of the experiment. In fact, studies indicate a great influence of the P450 complex, which is present in fungi of the genus *Penicillium*, in the degradation of contaminants (Szewczyk et al., 2020; Singh et al., 2024). No peaks were found to indicate the presence of the hydroxyatrazine metabolite, which further confirms the hypothesis that *P. chrysogenum* uses atrazine as a carbon source and performs the dealkylation metabolic pathway.

Biomass and sporulation evaluation by free *P. chrysogenum* NRRL 807

The biomass evaluation in the tests performed with free *P. chrysogenum* NRRL 807 was analyzed qualitatively (Figure 6) and gravimetrically with immobilized *P. chrysogenum* NRRL 807 (Figure 7). Free *P. chrysogenum* NRRL 807 demonstrated a different growth pattern for each nutrient source analyzed (Figure 6). In the treatment with the addition of exogenous sources of carbon and nitrogen and atrazine (ATZ+CN, Figure 6A), the mycelial growth was noticed in the form of more compact pellets. Regarding the treatment with the addition of an exogenous carbon source (ATZ+C, Figure 6B), the pellets can be seen as slightly larger, with hyphae. In the treatment with the addition of a nitrogen source (ATZ+N, Figure 6C), on the contrary, the pellets appeared to be even larger, presenting a more developed mycelial structure.

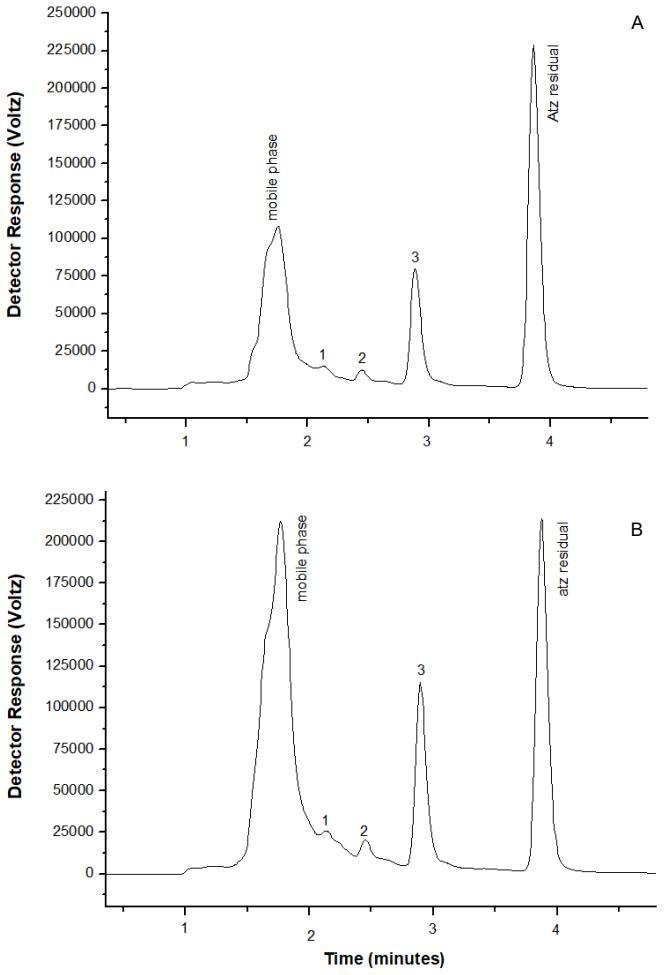

Figure 5 – Residual atrazine and metabolites verified at the end of the degradation tests (5 days) with (A) free and (B) immobilized P. *chrysogenum* NRRL 807. The numbered peaks refer to the degradation metabolites.

Finally, in the treatment without the addition of an exogenous source (ATZ, Figure 6D), there was a smaller amount of cellular structures, which did not appear to have a very well-defined shape.

In submerged cultures, many factors contribute to the appearance of a certain morphology in filamentous fungi, one of which is the availability of nutrients in the environment (Papagianni, 2004). The fungi may grow in the form of pellets or mycelial filaments that are dispersed in the medium. In the ATZ+CN medium, all nutrients were available. Thus, maintaining a more compact pellet may have protected it from the atrazine. In ATZ+C and ATZ+N treatments, only one of the nutrients was available. Consequently, the fungus had to search for additional sources of nutrients, which were present in the atrazine dissolved in the medium. As a probable response to this requirement, the fungus extended hyphae from the pellet to access these nutrients.

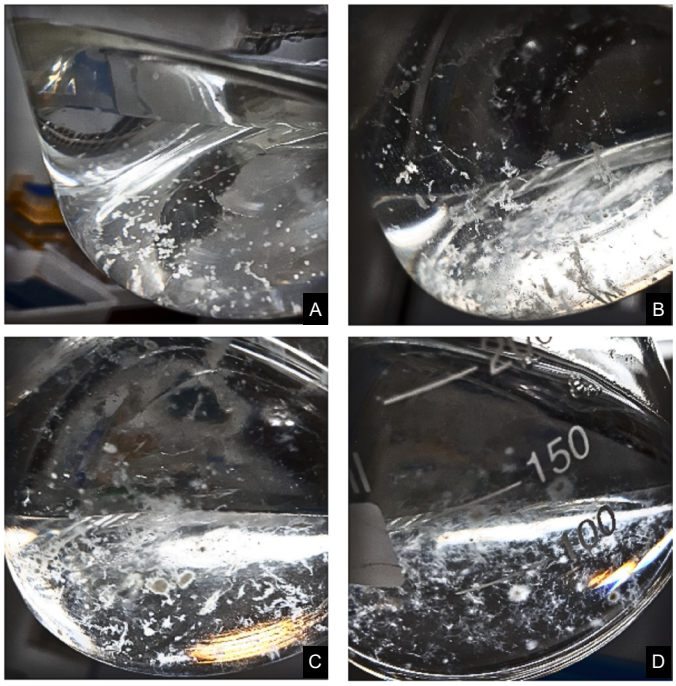

Figure 6 – Free P. *chrysogenum* NRRL 807 at the end of atrazine degradation tests (5 days) in minimal mineral medium with different nutrient substances: (A) ATZ+CN, medium with 25 mg/L of atrazine supplemented with 1.0 g/L glucose and 0.75 g $NaNO_3$; (B) ATZ+C, atrazine and glucose; (C) ATZ + N, atrazine and $NaNO_3$; and (D) ATZ, atrazine only.

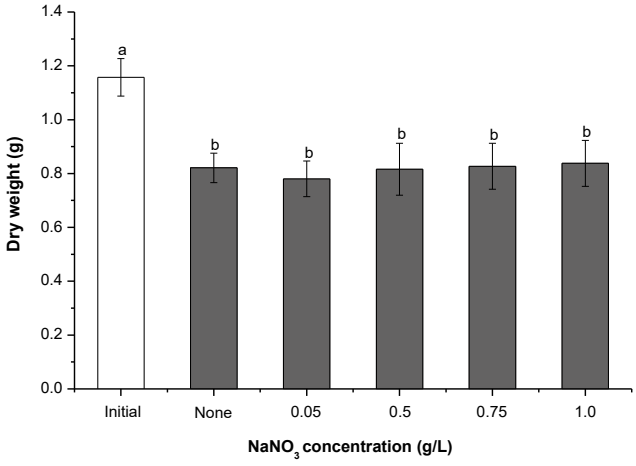

Figure 7 – Immobilized P. *chrysogenum* NRRL 807 dry weight (g) after 5 days of inoculation in Czapeq's medium (initial, white column) and after 5 days in minimum mineral medium with 25 mg/L atrazine with different source concentrations of exogenous nitrogen (dark columns). Error bars correspond to the mean values±standard deviation. Different letters in each period indicate statistical differences ($p<0.05$).

The pellet was less dense and, therefore, less protected (Böl et al., 2021). In the medium with only atrazine, the fungus was more exposed in an attempt to find the needed nutrients. This increased exposure may have promoted the process of cell lysis. The exhaustion of nutrients more easily as-

similated may have been one of the reasons for autolysis, responsible for the drastic change of pellets to the dispersed form. The limitation of nutrients increases the vacuolization of hyphae, making them more fragile and susceptible to damage by mechanical forces (Paul et al., 1994; Papagianni, 2004).

Biomass and sporulation evaluation by immobilized *P. chrysogenum* NRRL 807

In the evaluation of immobilized *P. chrysogenum* NRRL 807, a loss of mass was observed during the cultivation, in all the treatments (Figure 7). There were no significant differences in the mean values of the final dry mass in the different treatments with the addition of an exogenous nitrogen source. Biofilms confer several advantages to individuals in terms of their ability to withstand environmental stresses, such as the presence of toxic compounds (Harding et al., 2009). Due to the specific structural, biochemical, and physiological properties, these three-dimensional, biofilm matrices play a predominant role in the degradation of organic pollutants (Dash and Osborne, 2020). The extracellular polymeric substances (EPS) produced in biofilms reduce the transport and penetration of xenobiotics in cells, providing cellular protection (Córdova-Alcántara et al., 2019). The protection of cells located in the innermost layers of the biofilm is also recognized in the literature (Langer et al., 2018). The loss of mass observed may correspond to the loss of the outermost cells, less resistance to the toxicity of atrazine, and the damage caused by the limiting condition of nitrogen. In 10 days, in a preliminary test with a treatment without any addition of an exogenous nitrogen source, the maintenance of biomass was verified (0.89±0.20 g, according to the analysis of variance, $p<0.05$), indicating a possible resistance by the inner cells. However, there was no increase in the removal of atrazine. The loss of biomass also indicates that the immobilized *P. chrysogenum* NRRL 807 can protect itself from atrazine, using reserve sources. In other words, the immobilized *P. chrysogenum* NRRL 807 seemed to be able to use nutrients from its own mycelial tissue for maintenance, without having to use atrazine, protecting itself from its toxicity. The outermost cells of the biofilm, more exposed to the atrazine, probably used the herbicide as a carbon source.

Another factor observed was the occurrence of sporulation in some replicates in the different treatments analyzed. The higher the concentration of the exogenous nitrogen source, the higher the sporulation (in terms of DO_{600}) from the third to the fifth test day (Figure 8). This may explain the lack of growth (Figure 7) because the promotion of sporulation causes the inhibition of mycelial growth. Several studies relate the C/N ratio and sporulation, but the results vary according to the species and the source of nitrogen. Wang et al. (2023) verified that the sporulation in *Beauveria majiangensis* MJ1015 was significantly higher on media containing $NaNO_3$. Vu et al. (2019) also showed that $NaNO_3$ is a good source for promoting sporulation, as it is a good activator of the regulatory gene LaeA in *P. chrysogenum*. The results obtained in this study support that the increase in nitrate concentration also favored sporulation.

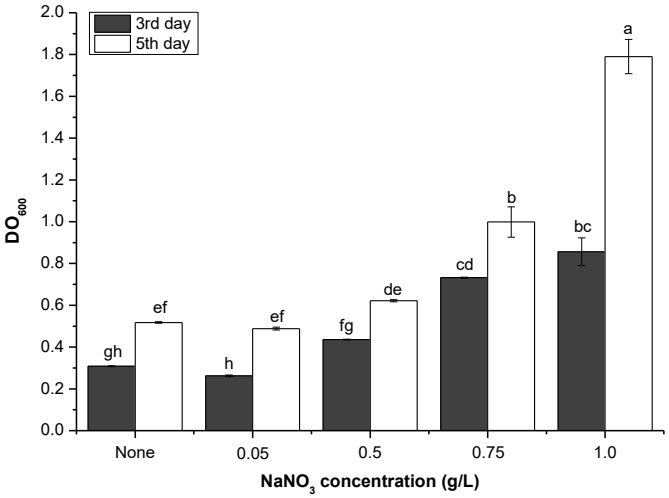

Figure 8 – Immobilized P. *chrysogenum* NRRL 807 sporulation (DO_{600}) after 3 and 5 days of test in the minimum mineral medium with 25 mg/L atrazine in different concentrations of the exogenous nitrogen source. Error bars correspond to the mean values±standard deviation. Different letters in each period indicate statistical differences ($p<0.05$).

Enzymatic activities

The enzymatic activities of laccase and MnP were evaluated with free and immobilized *P. chrysogenum* NRRL 807. There was no significant presence of extracellular enzyme activity in the process of removing atrazine from the medium in any of the treatments analyzed. It suggests that the degradation of atrazine in this study was not due to the action of extracellular enzymes.

The laccase and MnP enzymes are normally secreted by some microorganisms and are useful in the decomposition process of organic components. Several studies have highlighted the importance of these enzymes in the degradation of phenolic and amino aromatic contaminants. The laccases are enzymes that have copper in their structure and are capable of degrading various substances such as phenols and amino aromatics (Chen et al., 2019). However, they are known to be more efficient at degrading phenolic compounds than non-phenolic compounds due to their low redox potential. To compensate for this, it is common to use substances known as mediators that act by shuttling electrons. These mediators achieve this by oxidizing themselves with the help of enzymes and producing stable radicals (Saha and Mukhopadhyay, 2022). The MnPs are also capable of catalyzing oxidation reactions of various aromatic compounds. However, these reactions require the presence of divalent manganese (Su et al., 2024).

Few recent studies have indicated the real influence of free extracellular enzymes without mediators on atrazine degradation. Laccase from *Polyporus teiniculus* can be induced by increasing atrazine levels in the medium with high nitrogen content (Henn et al., 2020). At an initial concentration of 25 mg/L of atrazine, these authors verified an enzymatic activity of about 20 U/L. In our study, the extracellular enzyme activity that would be respon-

sible for the degradation of atrazine in the medium did not exceed 0.0009±0.0005 U/L. It is possible that the absence of a mediator capable of improving laccase efficiency, combined with the lack or insufficiency of essential substances necessary for forming the enzyme structures in the minimal mineral medium used, such as copper and divalent manganese, explains why the enzymes were unable to access the atrazine molecules.

Conclusions

Both free and immobilized *P. chrysogenum* NRRL 807 demonstrated great potential for the degradation of atrazine. The free *P. chrysogenum* was able to degrade 40% of the atrazine present in the medium after 5 days; the immobilized fungus degraded 48%. Both can be selected to compose bioremediation technologies, depending on the objective of the project and the location to be decontaminated. The small difference in the degradation efficiency between the tested forms indicates the ability of immobilized *P. chrysogenum* to protect itself from atrazine, using reserve nutrient sources for this. Notably, atrazine was used as a carbon source and the removal processes were led by the enzyme complex of cytochrome P450. The amount of exogenous nitrogen available interferes with the biodegradation efficiency, as, when in greater quantities, it diverts the route to the path of spore germination. Based on these results, *P. chrysogenum* both in its free form and when immobilized in biofilms can be used as bioremediation technologies for treating water contaminated by atrazine.

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Authors' contributions:

Silva-Nicodemo, S.C.T.: data curation, formal analysis, investigation, methodology, project administration, software, supervision, writing – original draft. Souza Filho, P.F.: data curation, formal analysis, methodology, writing – review & editing. Lima, M.M: methodology. Santos, E.S.: formal analysis, funding, resources, supervision, validation. Macêdo, G.R.: resources, supervision.

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