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Full Length Research Paper

Isolation of filamentous fungi present in swine wastewater that are resistant and with the ability to remove atrazine

Morgana S. Gonçalves[1]*, Silvio C. Sampaio[2], Luciane Sene[2], Floriano L. Suszek[2], Silvia R. M. Coelho[2] and Claudia E. C. Bravo[1]

[1]Coordination of Environmental Engineering Course, Federal University of Technology - Paraná, Francisco Beltrão, Paraná, Brazil.
[2]Postgraduate Program in Agricultural Engineering, Western Paraná State University, Cascavel, Paraná, Brazil.

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In this work, we isolated and identified filamentous fungi present in swine wastewater (SW) that are resistant and with the ability to remove atrazine removal. For the isolation, the SW was inoculated into liquid medium containing 0.01 and 0.1 g L^{-1} of atrazine and after the adaptation period, was transferred on solid medium containing 10 mg L^{-1} atrazine. Three strains of filamentous fungi were isolated and identified as *Cladosporium cladosporioide*, *Rhizopus stolonifer* and *Penicillium purpurogenum*. The isolates were tested for their ability to degrade atrazine in liquid medium containing 1.5 g L^{-1} of the herbicide as single source of carbon and nitrogen. In 15 days of culture, the isolated *R. stolonifer* showed a higher removal rate (73.75%), followed by *P. purpurogenum* (73.42%) and *C. cladosporioides* (68.42%), indicating a high possibility of using these microorganisms in the removal of atrazine in waters and effluents.

Key words: Atrazine, swine, filamentous fungi.

INTRODUCTION

Atrazine is a triazine herbicide used to control pre and post emergent grass and broadleaf in the crops of maize, sorghum and sugar cane (Correia and Langenbach 2006). Pesticides have been used in agriculture with the aim to get greater productivity in crops. However, only small amounts of the released agrochemical field reach the specific target, while the rest of the application has the potential to move into the soil and may reach surface and groundwaters. Results of some studies revealed the presence of atrazine alarming levels and its degradation products in soils and surface and groundwaters (Hallberg, 1989; Claver et al., 2006; Hildebrandt, 2008).

The atrazine molecule (2-chloro-4-ethylamine-6-isopropylamine-s-triazine), consisting of a N-alkylated and chlorinated heterocyclic aromatic ring (Figure 1), is not easily biodegraded, but microorganisms have demonstrated the ability to metabolize the molecule partially or completely, leading to the formation of NH_3 and CO_2 (Ueta et al., 2001; Wackett et al., 2002).

Technologies that use biological systems for biodegradation of hazardous waste can be applied in the decontamination of waters and effluents. Bioremediation processes usually use bacteria and fungi for biological degradation; in this case, the fungi protect itself of autoprotege pollutants, degrading them out of the cell wall, by the secretion of enzymes that catalyze the production of hydroxyl radicals and other reactive

*Corresponding author. E-mail: morgana@utfpr.edu.br. Tel./Fax: +55 46 3523 7111.

Abbreviation: SW, Swine wastewater.

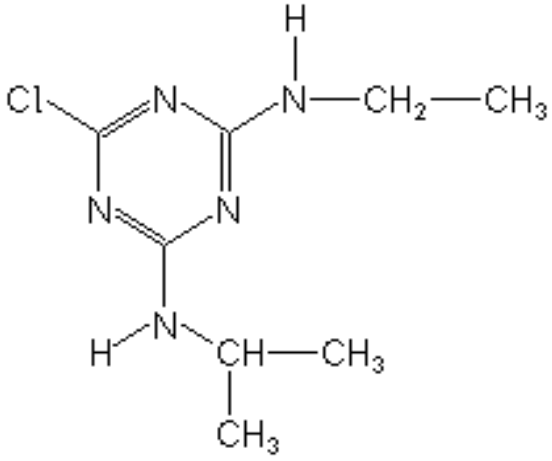

Figure 1. Atrazine molecular structure (2-chloro-4-ethylamine-6-isopropylamine-s-triazine).

chemicals. Since the hydroxyl radical in particular is rather non-specific with respect to the substance to be oxidized, fungi useful in degrading waste mixtures comprise various chlorinated substances (Baird, 2002).

In the microbiological context, the swine wastewater has some microbial diversity including fungi and bacteria, where some of these microorganisms have a high capacity for metabolism and broad spectrum of tolerance to adverse environmental factors (Wiecheteck et al., 2004). Therefore, the isolation and selection of microbial strains present in swine wastewater, with the ability to remove atrazine and the possibility of use in waters and effluents treatment, is an interesting work in areas submitted to intensive agriculture.

Considering the real scarcity of studies in literature that made the isolation of wastewaters microorganisms that have pesticides resistance and removal capacity, this study was intended to isolate and identify filamentous fungi present in the SW aiming its use in atrazine removal techniques.

MATERIALS AND METHODS

Swine wastewater

The swine wastewater was collected in a farm located in the city of Toledo, Paraná, Brazil, treated by biodigester followed by sedimentation tank, two stabilization ponds, tank of algae and fish tank. Samples of 500 ml were collected in sterile bottles in the exit of the first stabilization pond; two collections were made, one for each batch of the experiment.

Liquid medium

The composition of mineral solution (per liter of distilled water) for the first batch of tests, according to Vargha et al. (2005), was as follows: K_2HPO_4, 0.8 g; KH_2PO_4, 0.2 g; NaCl, 0.5 g; $MgSO_4$, 0.1 g; $CaCl_2$, 0.4 g; $FeSO_4$, 0.02 g and $MnSO_4$, 0.01 g.

For a better initial adaptation of microorganisms to medium containing atrazine, a second batch of tests under different conditions was done, however with the same composition of the cited culture medium, with the addition of NH_4H_2PO 41.73 g and 2.5 ml glucose (40%).

Atrazine

Atrazine used was commercial Atranex 500 SC® with a concentration of 500 gL^{-1}.

First bach

For the first batch of tests, 250 mL Erlenmeyer flasks were used containing 100 ml of mineral solution and atrazine in the concentration of 0.01 gL^{-1} as single source of carbon and nitrogen. Ten bottles were inoculated with 1 ml of swine wastewater. The incubation continued for 15 days at 100 rpm and temperature between 25 and 30°C. After this period, we transferred 0.1 ml of the medium in the Erlenmeyer flasks to Petri dishes containing the same mineral solution and concentration of atrazine plus 1.5% agar-agar. Five plates of each Erlenmeyer flask were subcultured and incubated at 28°C for 72 h, with the intention of observing the possible growth of resistant microorganisms to the presence of atrazine.

Second batch

In the second batch of tests, we used the same numbers of bottles and mineral solution, however, monobasic ammonium phosphate and glucose were added to increase the concentration of atrazine to 0.1 gL^{-1}. Incubation of the bottles followed at 150 rpm and 28°C for 15 days, and every seven days, the Petri dihes were subcultured.

Isolation and identification of filamentous fungi resistant to atrazine

After incubation of Petri dishes prepared in the first and second batch, the growth of resistant microorganisms was observed. The filamentous fungi that showed better growth were isolated and maintained on solid medium composed of mineral solution, atrazine (10 mgL^{-1}) as the single source of carbon and nitrogen, with agar (1.5%). The isolates were identified at the Osvaldo Cruz Foundation, from the microscopic observation of the structures in microcultivation.

Removal test in liquid medium

The filamentous fungi isolated were tested for their ability to remove atrazine. The isolates were grown in 150 ml of liquid medium containing the mineral solution as described above and atrazine in the concentration of 1.5 gL^{-1}, as the single source of carbon and nitrogen. The experiment was performed in triplicate; each bottle received 1.5 $x10^8$ spores of each fungus tested. After 15 days of cultivation, samples of the liquid medium was collected, filtered in membrane with 0.22 µm pore size and analyzed for the concentration of atrazine, using high performance liquid

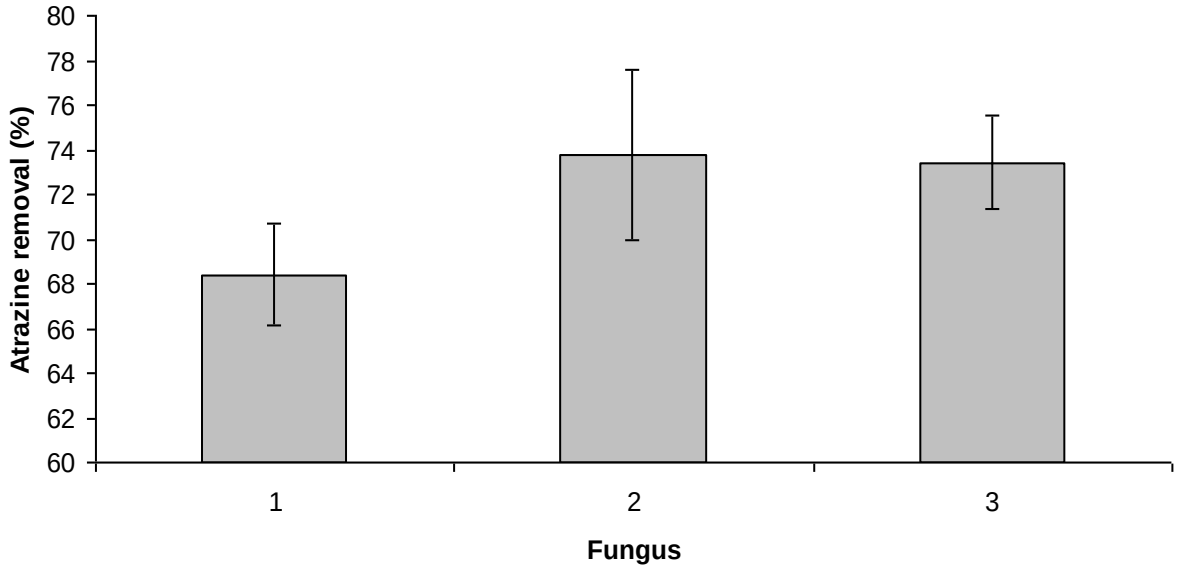

Figure 2. Atrazine removal by the fungi. (1) *C. cladosporioide;* (2) *R. stolonifer*e; (3) *P. purpurogenum*. Error bars indicate standard deviation, as calculated from three replicates.

chromatography (HPLC) under the following conditions: column C-18 (150 x 4.6 mm), mobile phase of methanol:water (50:50, v/v), UV detector at 230 nm, continuous flow of 1 ml min^{-1}, oven temperature of 35°C, runs of 15 min and injection volume of 20 µL.

The removal rate of atrazine was calculated from the difference between the initial concentration of the herbicide and after the incubation period.

RESULTS AND DISCUSSION

Three strains of filamentous fungi were isolated from swine wastewater (F1, F2 and F3) that were resistant to the presence of atrazine, and were identified as *Cladosporium cladosporioide*, *Rhizopus stolonifer*e and *Penicillium purpurogenum*, respectively.

Species of filamentous fungi of the genera *Cladosporium* sp., *Rhizopus* sp. and *Penicillium* sp. have been isolated from contaminated soils and residues, which demonstrated high capacity for degradation of pesticides (Bordjiba et al., 2001; Kodama et al., 2001; Liu et al., 2004; Martinez et al. 2008), aromatic hydrocarbons (Cerniglia 1997; Silva and Monteiro, 2000), oil (Pereira et al., 2004), among other xenobiotics. Isolates of *Penicillium* sp. and *Rhizopus* sp. with little sensitivity to atrazine and partial degradation of the molecule were reported by Ueta et al. (2001) and Colla et al. (2008).

The use of fungi in the removal of pollutants began to be studied in the last 30 years of the twentieth century (Soares et al. 2011) and by presenting biodegradation efficiency of a wide variety of organic compounds, the fungi are considered promising organisms in generating positive results in the bioremediation of degraded areas (Singh 2006). Pathak and Dikshit (2011) showed the use of biosorbents as a technology treatment for the removal of atrazine in water, wastewater and contaminated soils, and highlighted how important fungal biosorbents the genera *Aspergillus*, *Penicillium* and *Rhizopus*.

The filamentous fungi isolated in this study showed high atrazine removal efficiency (Figure 2), since the fungus *R. stolonifer* showed a higher removal rate (73.75%), followed by *P. purpurogenum* (73.42%) and *C. cladosporioide* (68.42%).

The ability to remove atrazine demonstrated by filamentous fungi isolated from swine wastewater (SW), indicates the possibility of using these microorganisms in triazine herbicides removal studies and other pollutants.

Studies have realized the isolation of fungi from waste and industrial effluents, aiming its use in remediation processes of contaminated areas. Chen et al. (2011) isolated a fungus of the genus *Cladosporium* from activated sludge from an aerobic pyrethroid-manufacturing wastewater treatment system. The isolate was shown to degrade 90% of fenvalerate, fenpropathrin, β-cypermethrin, deltamethrin, bifenthrin, and permethrin (100 mg L^{-1}) within 5 days. In this same context, Cortés et al. (2002) isolated the fungus *Rhizopus nigricans*, native from a paper mill effluent, studied its pentachlorophenol (PCP) tolerance and degradation capacity in solid-state culture. This fungus displayed a high tolerance to grow in the presence of PCP and degraded 60% of the compost in 24 hours.Several microorganisms among bacteria, yeasts and fungi have been isolated from soil contaminated with xenobiotics, aiming the development of techniques for decontaminating areas. Generally,

contaminated soils act as selective medium for microorganisms present in the area which are suitable and able to use the pollutant as a source of nutrients for their development.

Studies have shown that fungi isolated from soil, the same genus of filamentous fungi found in this study (*Cladosporium, Penicillium* and *Rhizopus*), have the ability to degrade several pollutants.

In an experiment conducted by Krivobok et al. (1998), the fungi *Rhizopus arrhizus* and *Cladosporium herbarum*, isolated from soil samples were able to degrade 95 and 85% of anthracene, respectively. Kodama et al. (2001) isolated from soil samples, the fungus identified as *Penicillium steckii*, which had a rate of degradation of simazine (herbicide s-triazine) of 53% in 5 days of culture in liquid medium at 30°C. Ouahiba et al. (2009) isolated fungi from contaminated soils by different types of herbicides and tested for their ability to biodegrade. One of the isolate, of the species *Rhizopus oryzae*, showed removal of 40% of the herbicide metobromuron in liquid medium. In a study conducted by Potin (2004), the fungus *Cladosporium sphaerospermum* was isolated from soil of an aged gas manufacturing plant. This strain was tested for its ability to degrade polycyclic aromatic hydrocarbons (PAHs), and showed an average of 23% biodegradation, including high molecular weight PAHs.

Technologies use microrganisms in the treatment of soils, residues, waters and effluents, making them less dangerous and impactful. Therefore, it is necessary to carry out further research to isolate new microrganisms potentially capable of adaptation to extreme environments and removal of pollutants, and to develop techniques for the remediation of degraded areas and treatment of waters and wastewaters.

Conclusion

The results demonstrate the existence of filamentous fungi capable of removing atrazine in swine wastewater, indicating the possibility of using these microrganisms in the treatment of waters and effluents.

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