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DEGRADATION OF 2,4-D HERBICIDE BY MICROORGANISMS ISOLATED FROM BRAZILIAN CONTAMINATED SOIL

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SHORT COMMUNICATION

ABSTRACT

The aim of this work was to isolate microorganisms from Brazilian soil contaminated with 2,4-D herbicide, and analyze the efficiency for 2,4D degradation, using high-performance liquid chromatography (HPLC). *Serratia marcescens* and *Penicillium* sp had never been reported as able to degrade 2,4-D. The isolated strains represent a great potential for bioremediation.

Key words: Bioremediation, Biodegradation, 2,4-dichlorophenoxyacetic acid.

2,4-dichlorophenoxyacetic acid (2,4-D) is an organic acid with pKa of 2.6 and high water solubility (45 g L^{-1}). It presents a systemic mode of action and has been widely employed in wheat, rice, corn, sorghum and sugar cane cultures to control harmful wide-leaf weeds (21). Particularly in Brazil, this herbicide is extensively used in many crops (16). Because it is highly selective and systemic, this herbicide is transported through the plant, being accumulated in the growing roots, inhibiting the growth of weeds.

2,4-D is classified by both ANVISA (Brazilian National Agency for Sanitarian Vigilance) and WHO (World Health Organization) as a hormonal herbicide of level II toxicity. It is considered a carcinogen agent, affecting liver, heart and central nervous system, leading to convulsions (6,13). This herbicide is usually commercialized as salt, amine and ester formulations, and has post-emergence action. After its application in field, the excess of the herbicide is easily transferred to the groundwater, due to its high solubility in water (600 mg.L^{-1} at 25ºC) (18). Even after a long period of disuse, considerable amounts of either 2,4-D or its main product of degradation, 2,4-dichlorophenol (2,4-DCF) (1), might be found in surface waters, and groundwater as well. Therefore, the development of an efficient degradation process for this herbicide is extremely relevant and necessary (10). The efficiency of the degradation methodology can be monitored and easily determined by using high-performance liquid chromatography (HPLC), since this technique allows the determination of the amount of herbicide and its metabolites in the samples (2).

2,4-D has frequently been used as a chemical model to investigate the evolution and diversity of catabolic genes involved in the degradation of anthropogenic contaminants in the environment. Although the chemical structure of 2,4-D is relatively complex, it is readily degraded and used as a carbon source by various environmental microorganisms (12).

Many investigations have suggested bioremediation as an alternative process for the removal of xenobiotic compounds from the soil, due to the smaller environmental impact, and greater efficiency (7,9,15,19,22).

The major aim of this work was to explore the metabolic versatility of microorganisms, and identify pure microbial

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cultures highly specialized in the degradation of the largely employed herbicide 2,4-D.

Soil samples were collected from wheat (*Triticum* sp) cultivation area (Capão da Onça Farm School – Universidade Estadual de Ponta Grossa, Paraná, Brazil - 25º 16'S; 50º 16'W), where the herbicide had been regularly used for a period of three years. The most recent application has occurred four months before collecting. About 50 g of soil were collected at ten different places, approximately 10 cm depth. The soil samples were thoroughly mixed, and portions of 1 g were further isolated and homogenized using a glass rod. In order to select the herbicide tolerant microorganisms, a serial dilution assay was carried out in 0.9% NaCl solution, which revealed that 10^{-3} was the best dilution for obtaining isolated colonies. They were plated in a rich medium, nutrient agar, and in a selective medium, nutrient agar plus 806 g/L of 2,4-D. The applied concentration of 2,4-D herbicide was equivalent to what is recommended for field experimentation. Inocula with soil suspension of 100 µL were used for each 1 g of sample.

In order to obtain mesophilic strains of different growing velocities, the cultures were incubated at 18 and 28ºC, in duplicates. After incubation, growing 2,4-D tolerant colonies were selected and stored in rich and selective medium, as well as in criogenic tubes, according to Castellani (3).

The quantity of 2,4-D herbicide in the supernatant of bacterial cultures was measured by HPLC, using a Shimadzu LC–10 Ai chromatograph, equipped with an UV detector. The separation was carried out in a Merck C-18 column (150 mm × 2.1 mm × 5 µm), and the elution was performed with methanol / 20 mM ammonium acetate (pH 4.5) - (50:50 / v:v), under a flow rate of 0.7 mL min^{-1}. The selected wavelength for 2,4-D detection was 230 nm.

The extension of the 2,4-D degradation process for each microorganism was accessed by monitoring the decrease of the 2,4-D peak area after incubation. For each evaluated condition, a control sample was prepared exactly in the same conditions, but with no microorganism. 2,4-D peak area achieved for the control samples was considered as 100%, and the degradation values obtained from the treatment samples were calculated based on the control value.

Five strains and the control were incubated for two weeks at 28ºC, in 100 mL of culture medium containing 2,4-D (2.35 mg mL^{-1}). The tubes were centrifuged at 12,100xg for 5 min, and an aliquot of 500 µL of the supernatants was submitted to purifying procedures, using C18 cartridges (100 mg Amersham/Amprep). The cartridges were washed gently with 1 mL of methanol and 1 mL of 0.5% TFA (trifluoroacetic acid) solution. The cartridges were then washed with 2 mL of 0.5% TFA. Finally, the sample was eluted with 10 mL of methanol, and 10 µL were submitted to HPLC. After the complete procedure, the final concentration of 2,4-D was 100 mg mL^{-1} for the control (Nutrient Broth plus 2,4-D).

The bacterial degrading strains were identified and characterized by Gram staining and biochemical assays. Lactose fermenter bacterial strains were selected in MacConkey agar and tested for decarboxylation of L-tryptophan, glucose fermentation, gas production from glucose, sulfidric gas production, urea hydrolysis, lysine and ornithine decarboxylation, motility, indol production, rhamnose fermentation, and citrate utilization, using Enterobacteria Kit (Laborclin, Pinhais, Brazil). The non-lactose fermenter isolates were tested for glucose fermentation/oxidation, growth in BHI broth and cetrimide, gelatinase production, motility, use of nitrate, xylose, maltose and lactose, using NF-PROV Kit (Newprov, Pinhais, Brazil). Capsule production was investigated by Gins Method (17). Fungal cultures were stained with lactophenol cotton blue and submitted to morphologic identification (11).

Among 25 isolated strains, five presented major degrading potential, and were identified as *Acinetobacter* sp, *Serratia marcescens, Stenothrophomonas maltophilia*, *Flavobacterium* sp and *Penicillium* sp. All these bacterial strains presented capsule, and were Gram negative. Capsule production could optimize the formation of a microbial consortium, where each strain would be responsible for the production of different enzymes which could increase herbicide degradation potential. The growth curve in the presence of 2,4-D demonstrated that the adaptation of the strains to the culture medium was very fast, with no lag phase. On the other hand, the curves also indicated fast growing, and therefore a good adaptability of these strains to media with herbicide, considering that log phase varied between 10 and 20 h. In order to determine the extension of the degradation by each isolated strain, samples and control solutions were analyzed by HPLC. The bacterial degradation of 2,4-D generated just one retention time peak in this approach. For this reason, the degradation efficiency was determined based on 2,4-D peak variation. Using 2,4-D peak area values obtained for samples and respective controls, the degradation values for each strain were: *Acinetobacter* sp – 0.25%; *Serratia marcescens* – 0.25%; *Stenothrophomonas maltophilia* – 30.20%; *Flavobacterium* sp – 10.10%; and *Penicillium* sp – 29.80% (Fig. 1).

The strains isolated in this work showed to be potentially 2,4-D degrading agents. *Acinetobacter* sp, *Stenothrophomonas maltophilia* and *Flavobacterium* sp had been previously described as 2,4-D herbicide degrading microorganisms in other studies (4,5,8,14,20), revealing a great importance in further studies aiming the bioremediation processes. The results are also noteworthy, considering that *Serratia marcescens* and *Penicillium* sp were isolated from tropical soil, and had never been reported as able to degrade 2,4-D before, a herbicide extensively used in Brazil.

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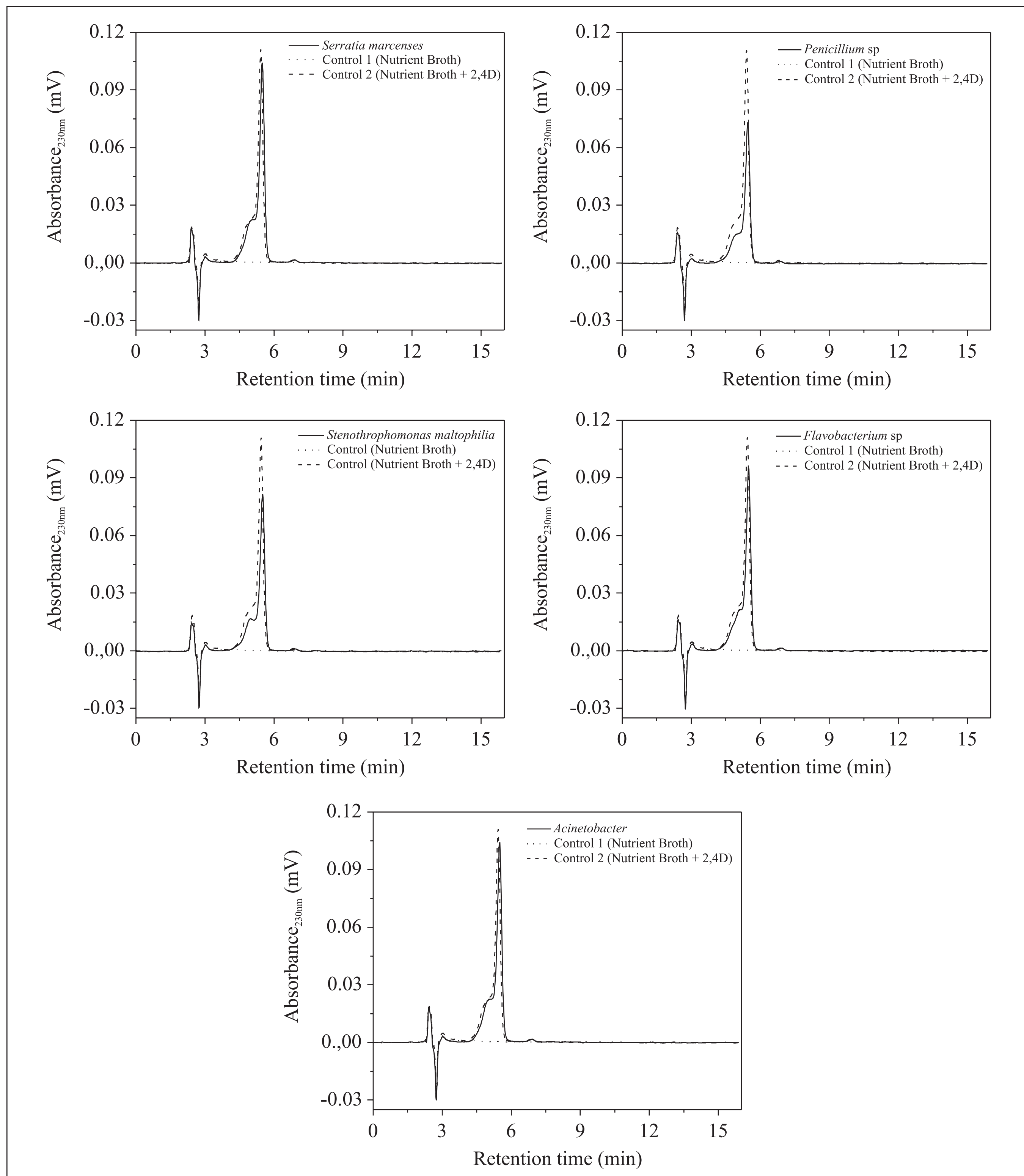

Figure 1. HPLC Analysis of 2,4-D biodegradation by: A) *Serratia marcescens*; B) *Penicillium* sp; C) *Stenothrophomonas maltophilia*; D) *Flavobacterium* sp.; E) *Acinetobacter* sp. and their respective controls, with no bacterium. The 2,4-D sample concentration in the control solutions after the purifying procedure was about 100 mg mL^{-1} in the control samples.

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RESUMO

Degradação do herbicida 2,4-D por microrganismos isolados de solo contaminado do Brasil

O objetivo deste trabalho foi isolar microrganismos de solo brasileiro contaminado com o herbicida 2,4-D, e analisar a eficiência da degradação por cromatografia líquida de alta eficiência (HPLC). *Serratia marcescens* e *Penicillium* sp jamais haviam sido relatadas como degradadoras de 2,4-D. As linhagens isoladas representam um grande potencial em biorremediação.

Palavras-chave: Ácido 2,4-diclorofenoxilacético, Biorremediação, Biodegradação.

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