An Acad Bras Cienc (2021) 93(Suppl. 4): e20210924 DOI 10.1590/0001-3765202120210924
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

MICROBIOLOGY

Pendimethalin biodegradation by soil strains of *Burkholderia* sp. and *Methylobacterium radiotolerans*

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Abstract: Pendimethalin herbicide is widely used and persists in the environment as a contaminant causing negative impacts, including for human health. Microorganisms have the capacity to remove many contaminants from the environment. Thus, the aim of this work was to evaluate the efficiency of soil bacterial species prospected by molecular modelling of cytochrome P450 in to degrade pendimethalin. Strains of *Burkholderia sp.* and *Methylobacterium radiotolerans* were cultivated in a mineral saline medium enriched with 281 mg/L pendimethalin (MSPEN) and another containing glucose 1.0 g/L as extra carbon source (MSPENGLI). Both strains were able to degrade pendimethalin under the two conditions experienced. *Burkholderia sp*. F7G4PR33-4 was more efficient in degrading 65% of the herbicide in MSPEN medium, with 49.3% in MSPENGLI; while *Methylobacterium radiotolerans* A6A1PR46-4 degraded 55.4% in MSPEN and 29.8% in MSPENGLI mediums. These findings contribute to the expansion of knowledge on the competence of isolates of these two bacterial genera in degrading herbicidal xenobiotics and biotechnological potential for pendimethalin degradation and bioremediation.

Key words: bioremediation, *Burkholderia*, *Methylobacterium radiotolerans*, pendimethalin biodegradation.

INTRODUCTION

The microbial process of transformation is considered as the major or sometimes the only means by which pesticides are eliminated from a variety of ecosystems. Microorganisms have the capacity to remove many contaminants from the environment by a diversity of enzymatic processes, many of them can be catalyzed by cytochrome P450 (CYP450) (Lovley 2003, Bernhardt 2006).

The widespread use and long persistence of dinitroaniline herbicides have resulted in their frequent detection in soil, groundwater and surface water, and these herbicides cause negative impacts on the environment. Pendimethalin is classified by the US Environmental Protection Agency (EPA) as a possible human carcinogen, and has been classified as a persistent bioaccumulative toxin (Hou et al. 2006, Roca et al. 2009, Huang et al. 2016).

Pendimethalin (3,4-dimethyl-2,6-dinitro-N-pentan-3-ylaniline) is an efficient selective herbicide from dinitroaniline class globally registered under several trade names, three in the Brazil, and used as selective herbicide recommended for pre-emergence weed control in acacia, cotton, garlic, peanut, rice, potato, coffee, sugarcane, onion, eucalyptus, beans,

tobacco, corn, pine, soy and wheat crops (AGROFIT/MAPA, Kole et al. 1994, Ni et al. 2016). Pendimethalin acts by disrupting mitosis in the meristematic cells of seedling plants by inhibiting the formation of microtubules (Strachan & Hess 1983, Mitra & Sept 2006). It is one of the two most widely used herbicide and the most used selective herbicide in the world with persistence in soil, reaching almost three years (Belden et al. 2003, Huang et al. 2016, Chen et al. 2021). Therefore, it is of interest the discovery of new efficient microorganisms for biodegradation of herbicides applicable in the bioremediation of contaminated areas.

In a previous study (Santos et al. in press) of molecular modeling to prospect for bacteria with potential for biodegradation of pendimethalin based on their affinity for CYP450, species of the Burkholderia and Methylobacterium genera are presented as potential biodegraders of this herbicide. Thus, this study objected to evaluate the efficiency of *Burkholderia* sp. and *Methylobacterium radiotolerans* soil isolates to degrade pendimethalin.

MATERIALS AND METHODS

Chemicals

Pendimethalin (3,4-dimethyl-2,6-dinitro-*N*-pentan-3-ylaniline) standard was obtained from Sigma-Aldrich (Germany). Dichloromethane HPLC-grade was obtained from JT Baker (United States). All other chemicals were of analytical grade.

Bacterial strains

Burkholderia sp. F7G4PR33-4 and *Methylobacterium radiotolerans* A6A1PR46-4 strains from the bacterial collection from the Laboratory of Soil Microbiology of the Embrapa Tabuleiros Costeiros - Brazil were evaluated for their ability to degrade and use pendimethalin as the sole carbon source.

Culture conditions and media

Luria Bertani (LB) medium (per liter): sodium chloride, 5 g; tryptone, 5 g; yeast extract, 2.5 g. LB medium was used to prepare the bacterial inoculum. Mineral saline (MS) medium [per liter of 20 mM phosphate buffer [(KH_2PO_4, Na_2HPO_4) pH 7.21]: Ca (NO_3)2.4H_2O, 0.6 g; $FeSO_4$.7H_2O, 0.2 g; $MnSO_4$.4H_2O, 0.02 g; $ZnSO_4$.7H_2O, 0.02 g; $CuSO_4$.5H_2O, 0.04 g; H_3BO_3, 0.003 mg; $NaMoO_4$.2H_2O, 0.004 mg; conc. H_2SO_4, 1 mL; $MgSO_4$, 0.2 g (Kröckel & Focht 1987). Glucose, tested as additional carbon source in an additional simultaneous culture, was added at final concentration of 1.0 g/L and ammonium sulfate at 0.5 g/L. The concentration of pendimethalin as carbon source in the culture media was 281 mg/L (1 mM).

The replicated cultures were grown aerobically in 125 mL Erlenmeyer flasks containing 50 mL of liquid medium in a rotary shaker at 120 rpm and 30° C. *Burkholderia sp.* F7G4PR33-4 and *Methylobacterium radiotolerans* A6A1PR46-4 strains were pre-inoculated in LB medium and incubated until the mid-exponential phase was reached after 12 h of culture (OD600 nm 0.5). After growth, cells from each flask were harvested by centrifugation at 5.000 g and the pellet was washed with 20 mM phosphate buffer to eliminate medium residues. Cells were resuspended in fresh MS with an adjusted density of 6.0×10^8 CFU/mL. An aliquot of the cell suspension (1% V/V) was added into 50 mL of MS 281 mg/L pendimethalin (MSPEN) and MS 281 mg/L pendimethalin plus glucose 1.0 g/L (MSPENGLI) mediums for analysis of pendimethalin biodegradation at 0, 4, 6, 10, 20 and 30 days of incubation. Bacterial growth was monitored based on the colony forming units (CFU/mL). Another non inoculated flasks containing 50 mL of MS 281 mg/L pendimethalin

and 281 mg/L pendimethalin plus glucose 1.0 g/L media set was prepared for abiotic control.

Extraction of pendimethalin from the cultures

The disappearance of pendimethalin was monitored by gas chromatography coupled to mass spectrometry (GC-MS). Therefore, aliquots of 1 mL from the cultures of each bacteria media were added to 10 mL with deionized water and extracted 3 times with 10 mL of dichloromethane, always collecting the lower organic phase. The organic layer was dried with anhydrous sodium sulfate and concentrated in rotaryt evaporator at 45 °C. The concentrated material was recovered in dichloromethane and transferred into a vial and kept in 1.0 mL of the solvent.

Biodegradation analysis

The chromatographic analyses of pendimethalin biodegradation were performed using a Shimadzu GC-MS QP2010Plus quadrupole gas chromatograph (Kyoto, Japan). A 30 m×0.25 mm NST 05-MS fused-silica capillary column with a film thickness of 0.25 mm obtained from Agilent was used. Helium (purity≥99.999%) was used as a carrier gas at a flow rate of 0.98 mL/min. A 1 µL extract was injected in a splitless mode at an injection temperature of 280° C. The oven temperature was programmed to increase from an initial temperature of 60° C (held for 1min), increased to 100° C with ramp of 25° C/min, then the temperature was raised to 250° C with ramp of 15° C/min, for ramp from 20° C to 300° C (held for 5min). The temperatures of the ion source and mass-selective detector interface were 260 and 300° C, respectively. The GC–MS system was operated in the selected-ion monitoring (SIM) mode with the electron multiplier tune value. The electron multiplier voltage for pendimethalin was set to 200V relative to the tune value. The 252 quantitative ion was monitored.

Statistical analysis

Microsoft Excel (2016) was used for data analysis. To assess the statistical differences from groups, the Tukey's multiple range tests and one-way ANOVA were used at probability of 0.05 or less was considered significant.

RESULTS

Solubility of pendimethalin in dichloromethane and water is >800 g/L (20° C) and 0.33 g/L (20° C), respectively. In this system, the low water-solubility of the pendimethalin enables to remove any co-extractives which are also soluble in water. The extraction method used only 1.0 mL of the sample, gave overall recoveries in the MS medium samples ranged from 83 to 120%, compared to the analytical standard at the same concentration in dichloromethane. This liquid-liquid extraction method employed in combination with the optimized chromatographic protocol for detection of pendimethalin residue from cultures resulted in the adequate preparations for quantification of degradation (Figure 1).

There were significant differences ($p < 0.05$) between the levels of pendimethalin at different culture conditions compared with the abiotic control (Table I). The results for *Burkholderia* sp. F7G4PR33-4 ability to degrade pendimethalin are illustrated in Figure 2. It is shown that in the MSPEN treatment the strain F7G4PR33-4 could degrade about 24.94±4.77% of pendimethalin within 10 days of incubation, and 65.03±13.04% pendimethalin within 30 days of incubation, with an accentuation in activity and increase in cell density from day 10. However, in the MSPENGLI treatment, the decrease in the concentration of pendimethalin was observed only from day 20, which reached 49.35±7.78% after 30 days, even with a small decrease in cell density.

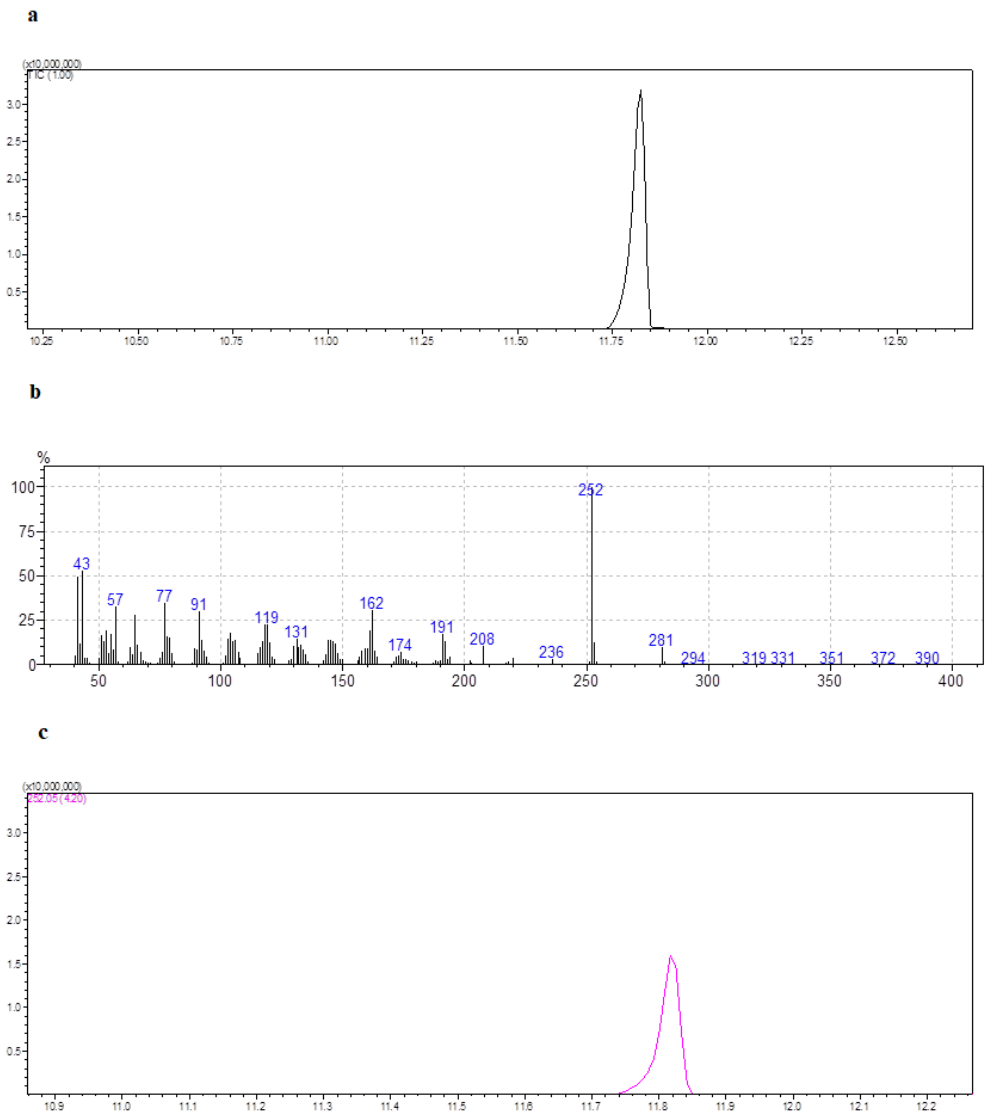

Figure 1. **Chromatogram of optimized method (a) and mass spectra (b) of the pendimethalin fragmentation by GC-MS showing structures of the molecular ion (281) and ion 252. Peak of the 252 ion used for quantification (c).**

For *M. radiotolerans* A6A1PR46-4 ability to degrade pendimethalin the results are in Figure 3. It's shown that in the MSPEN treatment the strain A6A1PR46-4 could degrade about 8.46±2.11% of pendimethalin within 10 days of incubation, and 55.46±2.54% pendimethalin within 30 days; and as for *Burkholderia sp.* F7G4PR33-4, a marked decrease concentration and increase in cell density was observed from day 10. In the MSPENGLI treatment, the decrease in the concentration of pendimethalin was observed only from day 20, which reached only 29.82±5.4% in 30 days, with an increase in cell density.

Evaluation of the pendimethalin loss by volatilization in the abiotic control media, also by CG-MS, showed that in 30 days there was a decrease in the concentration of 5.53±4.76% and 4.83±4.74% in the MSPEN and MSPENGLI media, respectively.

Table I. Concentration of Pendimethalin (mg/ml) following incubation with the bacteria strains in two mineral salt mediums and abiotic degradation controls after 30 days of culture.

Bacterial strains	Culture condition				
	MSPEN	MSPENGLI	ABIOTIC MSPEN	ABIOTIC MSPENGLI	MSD
	Mean ± S standard deviation				
M. radiotolerans A6A1PR46-4	126.33^{a}± 3.21	203^{b}± 10.81	265.33^{c}± 12.01	269^{c}± 12.76	22.16
Burkholderia sp. F7G4PR33-4	97.66^{a}± 12.74	143^{b}± 11.13	265.33^{c}± 12.01	269^{c}± 12.76	22.95

Means followed with the same letter in the same raw are not significantly different at $p < 0.05$ according to MSD (minimal significant difference).

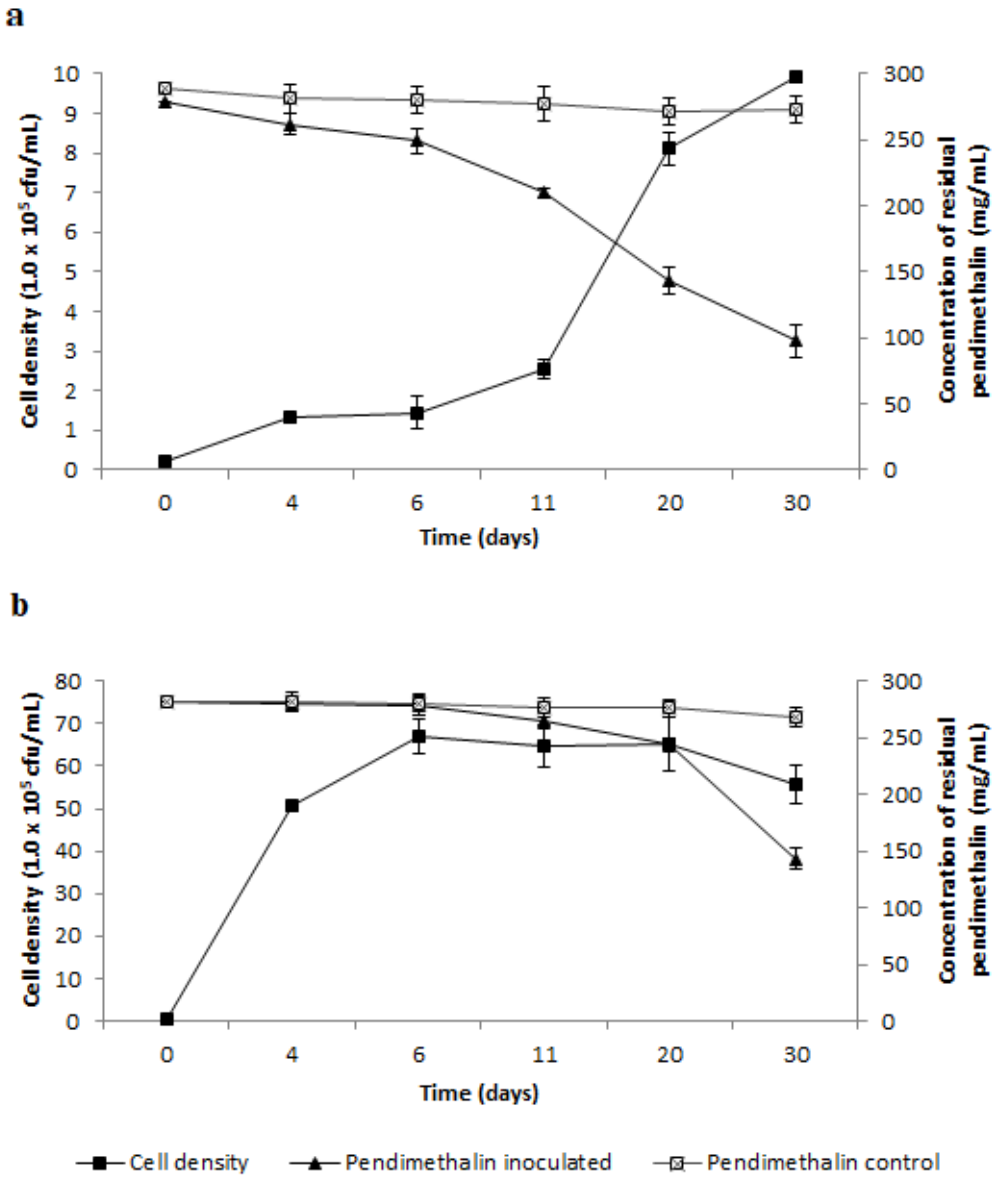

Figure 2. Biodegradation of pendimethalin by *Burkholderia sp.* F7G4PR33-4 strain in MSPEN (a) and MSPENGLI (b) mediums.

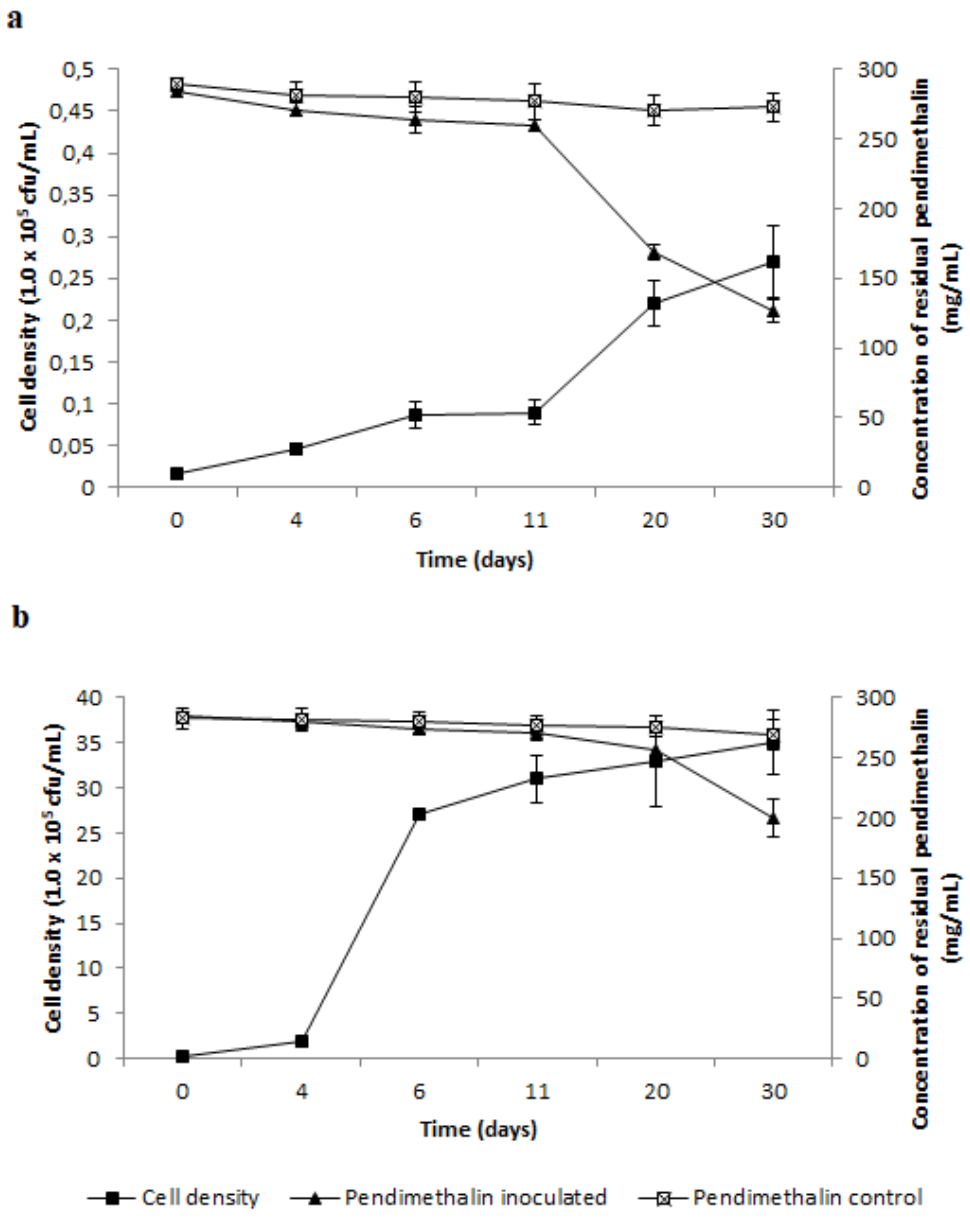

Figure 3. Biodegradation of pendimethalin by *Methylobacterium radiotolerans* A6A1PR46-4 strain in MSPEN (a) and MSPENGLI (b) mediums.

DISCUSSION

Burkholderia and *Methylobacterium* genera are members of the phylum Proteobacteria, respectively of the orders Burkholderiales and Rhizobiales, mainly found in soils and diverse parts of plants, including root-nodulating nitrogen-fixing symbiosis (Lidstrom & Chistoserdova 2002). Some strains with potential biotechnological application in bioremediation have been reported for degradation of recalcitrant xenobiotics, including pesticides, polycyclic aromatic hydrocarbon (PAH) and polychlorinated biphenyls (PCBs) compounds (Coenye & Vandamme 2003, Liang et al. 2014, Javaid et al. 2016, Onder Erguven et al. 2021). However, to date there has been no record of pendimethalin biodegradation by isolates of these two bacterial genera.

CYP450 from species of *Burkholderia* and *Methylobacterium* genera, previously studied for their theoretical affinity to pendimethalin (unpublished data), presented quali-quantitative similarities in interaction with the herbicidal compound in relation to that observed for human CYP1A1, which confers pendimethalin resistance in transgenic rice plant (Kawahigashi et al. 2007). Therefore, the decision to evaluate the degradability of pendimethalin by strains of the *Burkholderia* and *Methylobacterium* based on the theoretical affinity of their CYP450 with the herbicide was then correct, since cytochromes P450 are responsible for oxidation reactions in xenobiotics (Bernhardt 2006). In addition, Kole et al. (1994), Megadi et al. (2010) and Ni et al. (2016) reported the microbial pendimethalin degradation by oxidative N-dealkylation and nitroreduction, these reactions being of CYP450 competence.

Pendimethalin concentration of 281 mg/L, or 1 mM, applied in this study is in the intermediate range of the recommended for xenobiotic biodegradation studies (Focht 1994) and for those reported in previous studies by Sato et al. (1990) and Kole et al. (1994), that used respectively, 5 and 500 ppm. This concentration is still above 200 mg/L pendimethalin used by Ni et al. (2018) for degradation of pendimethalin by *Paracoccus* sp. P13, as well as 180 mg/L of pendimethalin tolerated by strains M-1 and LSMR-1 of *Rhizobium leguminosarum* and *Strenotrophomonas maltophilia* RB-3 under *in vitro* culture conditions performed by Singh & Singh (2020); which is higher than its residue concentration in the environment and has typically been used in biodegradation.

The biodegradation rates presented by isolates *Burkholderia sp.* F7G4PR33-4 and *M. radiotolerans* A6A1PR46-4 in the MSPEN medium are higher than those presented by Kole et al. (1994), which observed that 45% and 55% metabolism of pendimethalin caused by *Azotobacter chroococcum* after 10 and 20 days of incubation, respectively. Megadi et al. (2010) and Ni et al. (2016) reported efficiency of *Bacillus circulans* and *Bacillus subtilis* Y3 for >99% of pendimethalin degradation, even though the latter used a concentration almost three times lower than that of the present study.

In general, the behavior of the two strains was similar, since both showed greater degradation activity in the MSPEN medium with pendimethalin as the only source of carbon and energy to grow, although *Burkholderia sp.* F7G4PR33-4 had a hundred fold higher cell density than *M. radiotolerans* A6A1PR46-4. On the other hand, we observed a rapid increase in cell density for the two strains in the MSPENGLI medium with a decrease in the concentration of pendimethalin only near the end of the observation period, probably due to the phenomenon of biphasic catabolism, or diauxie, enhanced in its genetic bases by Traxler et al. (2006); where glucose is preferably used by cells.

CONCLUSIONS

The present study have shown that *Burkholderia sp.* F7G4PR33-4 and *Methylobacterium radiotolerans* A6A1PR46-4 were able to degrade pendimethalin and use it as the sole carbon source to grow in *in vitro* culture conditions. These findings contribute to the expansion of knowledge on the competence of isolates of these two bacterial genera in degrading herbicidal xenobiotics and biotechnological potential for pendimethalin biodegradation and bioremediation.

Acknowledgments

The authors would like to thank Embrapa Tabuleiros Costereiros for providing the bacterial strains and laboratory support, and the Brazilian Institutes

Fundação de Apoio à Pesquisa e à Inovação Tecnológica do Estado de Sergipe - FAPITEC/SE [grant number 8444.UNI332.22122.17102013/2013-02 (PPSUS Sergipe)]; Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq [grant number 446425/2014-1]; and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES [grant number 23038.010050/2013-04] for financial support.

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How to cite

SANTOS MIS, BRANDÃO ECTA, SANTOS E, BATISTA MVA, ESTEVAM CS, ALEXANDRE MR & FERNANDES MF. 2021. Pendimethalin biodegradation by soil strains of *Burkholderia* sp. and *Methylobacterium radiotolerans*. An Acad Bras Cienc 93: e20210924. DOI 10.1590/0001-3765202120210924.

Manuscript received on June 23, 2021;
accepted for publication on September 24, 2021

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Author contributions

MISS and MVAB conceived and designed research. MISS, ES and ECTAB conducted experiments. CSE, MRA and MFF contributed with reagents and analytical tools. MISS, MVAB, MRA and MFF analyzed data. MISS wrote the manuscript. All authors read and approved the manuscript.