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Insights into the microbial degradation pathways of the ioxynil octanoate herbicide

Karina O. Oliveira[a], Amanda R.M. Silva[b], Bianca F. da Silva[a], Humberto M.S. Milagre[a], Cintia D.F. Milagre[a,*]

[a] UNESP – São Paulo State University, Institute of Chemistry, Araraquara, 14800-060 SP, Brazil
[b] USP – University of São Paulo, Institute of Chemistry, São Paulo, 05508-000 SP, Brazil

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ABSTRACT

This paper describes the biodegradation of the ioxynil octanoate herbicide by indigenous microorganisms isolated from herbicide impacted soil-enrichment cultures. Eleven positive hits out of twenty-nine microorganisms screened for nitrile hydratase, nitrilase and amidase activity were further evaluated based on their growth in microtiter plates containing liquid medium with increasing concentrations of herbicide (0.97–250 mM). Two strains were selected from this assay for biodegradation studies and were identified as *Lysinibacillus boronitolerans* MLH-31 and *Bacillus cereus* MLH-61. The bacterial degradation of ioxynil octanoate and its biodegradation products were monitored, identified and characterized by liquid chromatography tandem mass spectrometry (HPLC-MS/MS). In addition to 3,5-diiodo-4-hydroxybenzamide and 3,5-diiodo-4-hydroxybenzoic acid, which are commonly detected metabolites, two new metabolites were observed: mono-deiodinated compound 3-iodo-4-hydroxybenzoic acid and the product of $C_{aromatic}$-CN cleaved 1,3-diiodophenol. The experimentally observed metabolites were correlated with the enzymatic systems involved, revealing the presence of esterases, nitrile hydratases, amidases, nitrilases, dehalogenases and carbon-carbon lyases during biodegradation. *Lysinibacillus boronitolerans* MLH-31 was found to degrade ioxynil octanoate at a rate of 97% over 7 days through a batch-resting cells experiment, while *Bacillus cereus* MLH-61 was found to do so at a rate of 75% under the same conditions.

1. Introduction

By 2050, the world's population will rise from today's 6.8 billion to 9.1 billion according to the latest United Nation projections, and at the same time food demand levels will increase, requiring roughly 70% more food while land and water resources will remain limited (Food and Agriculture Organization of the United Nations, 2016). To address this issue, a key strategy employed worldwide has involved the use of pesticides. However, the excessive use of pesticides is controversial, as they may threaten food, environmental and rural worker security because such compounds and/or their metabolites can be toxic and persistent to environmental, animal and human health (Holtze et al., 2007a, 2007b). Thus, determining the behaviors of pesticides in the environment and the impacts of these chemical species and their metabolites is of upmost importance and can support their more conscious use.

Benzonitrile herbicides have attracted attention from the scientific community given their global use on several crops since the 1970s to control weed growth in agricultural areas and given that some metabolites are persistent in the environment. High persistency levels of the dichlobenil metabolite 2,6-dichlorobenzamide in European aquifers have led to dichlobenil eradication in several countries, starting with Denmark in 1997 and followed by Brazil in 2002 and all European Union member states in 2011 (Clausen et al., 2006; Holtze et al., 2007a, 2007b; Pukkils et al., 2009; ANVISA, 2002; Rottherdan Convention, 2011). By far, ioxynil degradation is the least studied benzonitrile herbicide and less data are available on ioxynil octanoate (Holtze et al., 2008). Ioxynil and ioxynil octanoate are selective post-emergent contact herbicides used to control broad-leaved weed growth in crops by the inhibition of photosynthesis (Frkova et al., 2014).

The physical and chemical properties of ioxynil octanoate are shown in Table 1 in the Supplementary material. Based on its log P value, it presents hydrophobic rather than hydrophilic features that enable it to penetrate cell membranes and even show potential for

* Corresponding author.
E-mail addresses: kaotaoliveira@gmail.com (K.O. Oliveira), amandaribeiroms@yahoo.com.br (A.R.M. Silva), biancafs@iq.unesp.br (B.F. da Silva), humbertomilagre@iq.unesp.br (H.M.S. Milagre), cintiamilagre@iq.unesp.br (C.D.F. Milagre).

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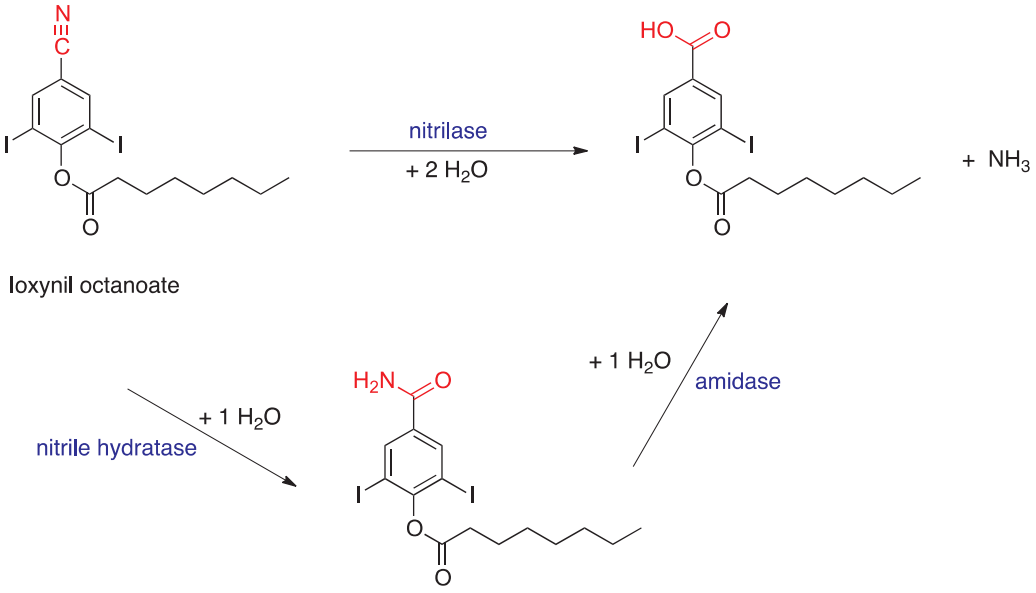

Scheme 1. Main enzymes involved in benzonitrile herbicide biodegradation.

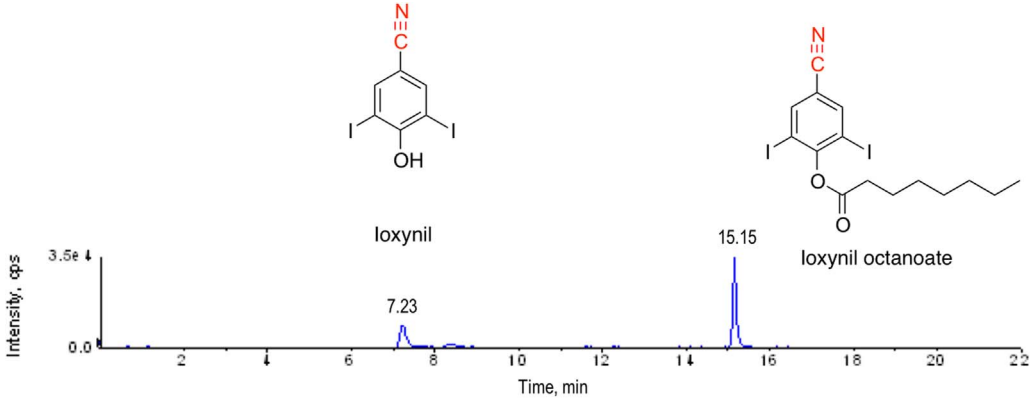

Fig. 1. Total ion chromatogram (TIC) of ioxynil at 7.23 min and ioxynil octanoate at 15.15 min.

bioaccumulation (European Commission, 2004). In humans, ioxynil has been reported to be a strong inhibitor of thyroid hormone-binding proteins interfering with the thyroid axis and to behave as an endocrine disruptor (Morgado et al., 2007). Moreover, it has been suggested that ioxynil could have moderate cytotoxic effects on two human cell lines (Hep G2 and HEK293T) through the liver and kidneys, respectively and that its amide and carboxylic acid metabolites are less toxic than the parent herbicides (Lovecka et al., 2015). A recent study that identified ioxynil octanoate residue in maize and soil revealed that its use in maize is safe, as no residue was detected at the time of harvesting after 60 days of pesticide treatment (Hu, Dong and Zhen, 2012).

It was observed that the abiotic degradation of benzonitrile herbicides in soils and sediments has minor effects relative to biodegradation (Holtze et al., 2006, 2008; Vesela et al., 2010). The main microbial enzymes responsible for ioxynil biodegradation, as is shown in Scheme 1, are the enzymes involved in nitrile metabolism: nitrile hydratase (EC 4.2.1.84), amidase (EC 3.5.1.4) and nitrilase (EC 3.5.5.1) (Vesela et al., 2012; Pasquarelli et al., 2015; Detzel et al., 2013; Nielsen et al., 2007, Grab et al., 2000).

Bacteria from the genera *Klebsiella*, *Rhizobium*, *Variovorax* and *Pseudomonas* are known to effectively degrade ioxynil to the supposedly less toxic metabolites 3,5-diiodo-4-hydroxybenzamide and 3,5-diiodo-4-hydroxybenzoic acid (Lovecka et al., 2015). While the complete deiodination of ioxynil to 4-cyanophenol by *Desulfitobacterium chlororespirans* bacteria has been observed under anaerobic conditions, no dehalogenation products from its correspondent amide and carboxylic acid metabolites have been experimentally observed thus far (Cupples et al., 2005; Holtze et al., 2008). The degradation of ioxynil to CO_2 by bacteria has only been described in reference to the *Klebsiella* strain (Holtze et al., 2008).

The aims of this study are to isolate bacteria of enrichment cultures from ioxynil octanoate exposed soils, to identify potential ioxynil degraders, to monitor the biodegradation of ioxynil octanoate through HPLC-MS/MS batch-resting cell experiments and to correlate the kinetics of experimentally observed metabolites with enzymatic pathways.

2. Materials and methods

2.1. Chemicals

Analytical grade ioxynil (CAS RN 1689-83-4), ioxynil octanoate (CAS RN 3861-47-0), and isobutyronitrile (CAS RN 78–82-0) were purchased from Sigma-Aldrich (São Paulo, Brazil). Nutrient broth (NB) and Agar were obtained from Acumedia (São Paulo, Brazil). Inorganic salts were purchased from Vetec (São Paulo, Brazil). HPLC grade and analytical solvents were purchased from Panreac (São Paulo, Brazil) and Synth (São Paulo, Brazil), respectively.

2.2. Microbial collection and isolation of bacteria

Twenty-nine bacteria strains isolated via the enrichment technique from soils previously exposed to Totril® herbicide (active ingredient: ioxynil octanoate) were used in the experiments (strains: MLH-29 to

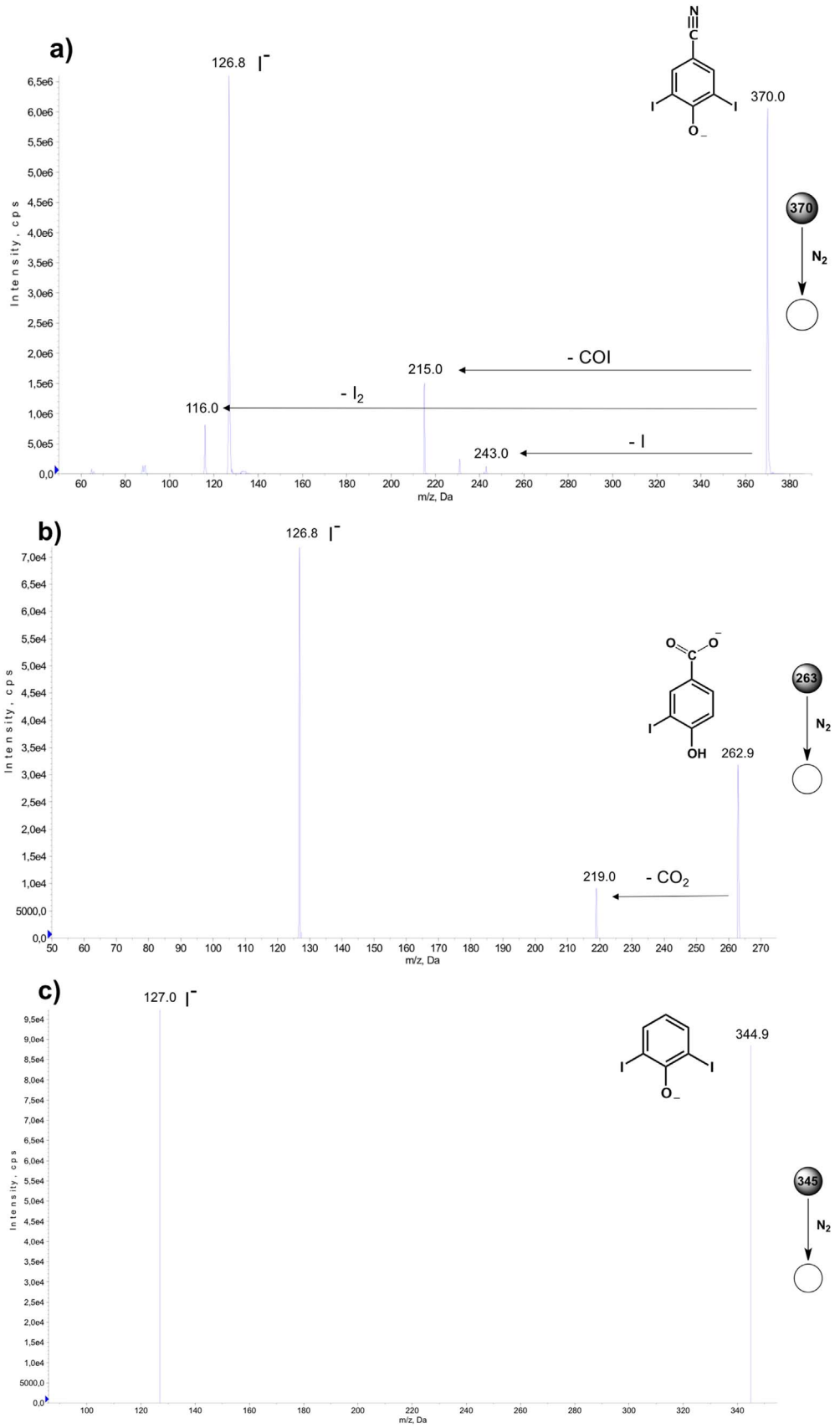

Fig. 2. ESI-(-)-MS/MS spectra for ions of a) *m/z* 370; b) *m/z* 263; c) *m/z* 345; d) *m/z* 388 and e) *m/z* 389.

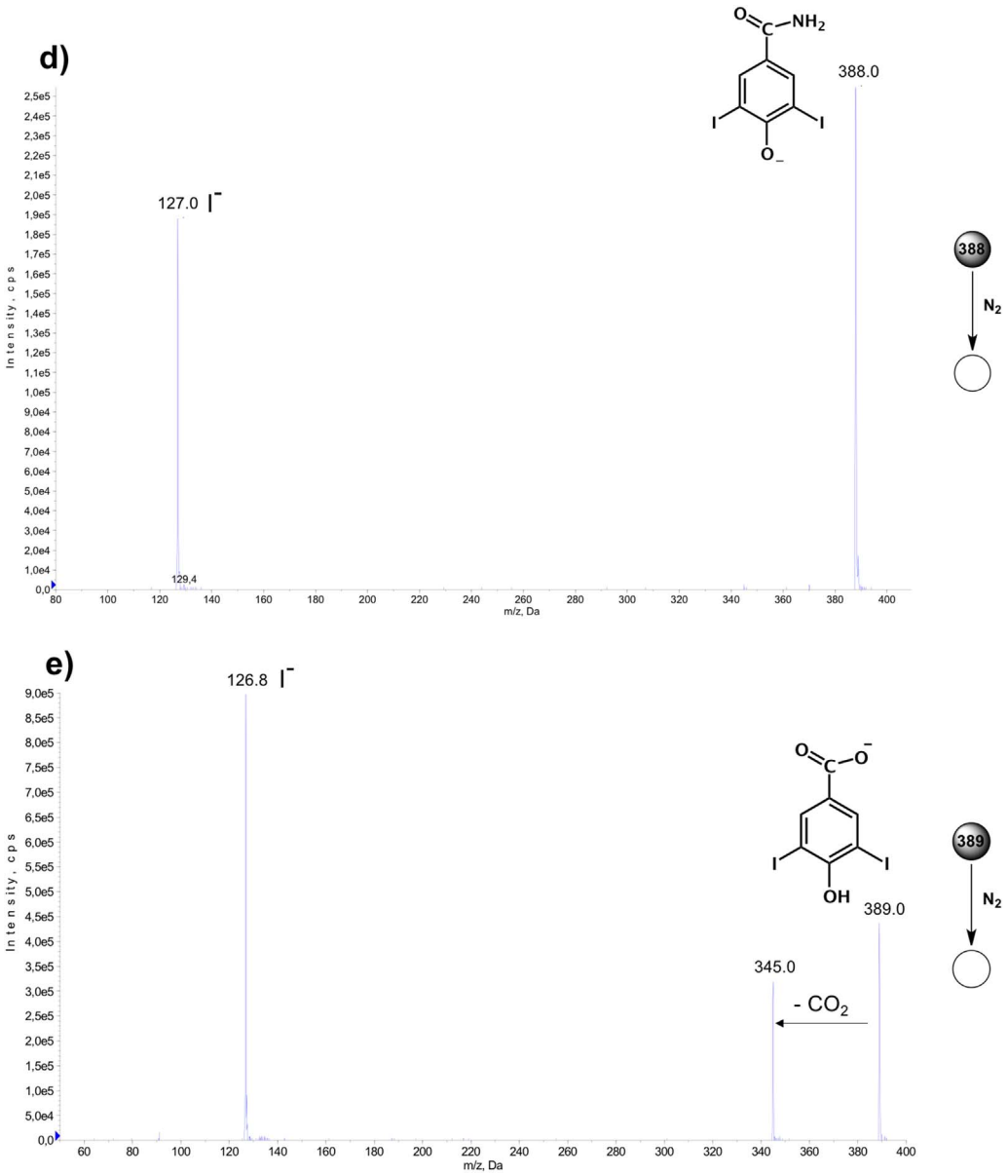

Fig. 2. (*continued*)

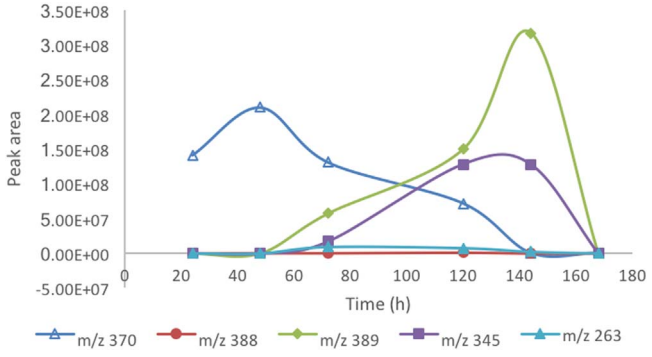

Fig. 3. HPLC-MS/MS monitoring of ioxynil octanoate biodegradation by *Lysinibacillus boronitolerans*-MLH-31 over 7 days (168 h).

MLH-40, MLH-42 to MLH-45, MLH-48, MLH-51 to MLH-57, and MLH-59 to MLH-63).

A mineral medium containing (g L^{-1}) $FeCl_3 \times 6H_2O$ (0.08), $CaCl_2$ (0.05), $MgSO_4 \times 7H_2O$ (0.02), Na_2HPO_4 (4.0), KH_2PO_4 (2.0) and glucose (1.8) was used for the enrichment culture. After being sterilized, the medium was supplemented with 1 mL L^{-1} trace metal solution, 1 mL L^{-1} vitamin solution and 10 mL L^{-1} Totril®. Trace element solution was composed of (g L^{-1}) $MnCl_2 \times 1H_2O$ (0.030), $NaVO_3 \times 1H_2O$ (0.003), $Na_2WO_3 \times 2H_2O$ (0.002), H_3BO_3 (0.005), $NiSO_4 \times 6H_2O$ (0.005), $ZnSO_4 \times 1H_2O$ (0.006), $CoCl_2 \times 6H_2O$ (0.004), $Na_2MoO_4 \times 2H_2O$ (0.002), $CuSO_4$ (0.003). Vitamin solution was composed of (g L^{-1}) biotin (0.1), calcium pantotenate (0.02), inositol (0.1), nicotinic acid (0.02). Microbial isolation was performed according to Sorokin et al. (2007).

All strains were maintained on nutrient Agar (NA) at 4 °C and were periodically transferred to a fresh medium (NB). The strains generating better results through biodegradation experiments were characterized via the Sanger sequencing of PCR products of amplified 16S rRNA gene, through outsourced services performed by BPI Genotyping (http://genotypingbpi.com.br - Botucatu – SP/Brazil).

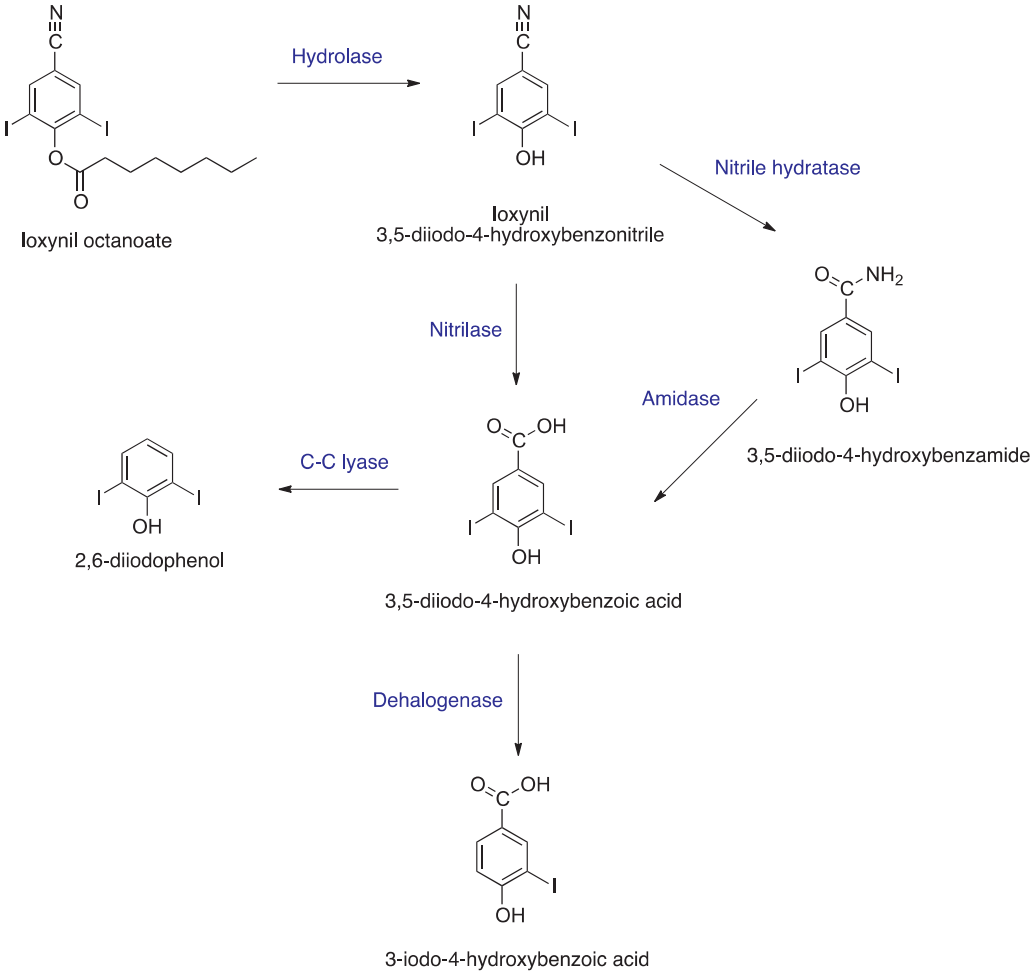

Fig. 4. Enzymatic pathways of ioxynil octanoate biodegradation by *Lysinibacillus boronitolerans* MLH-31 experimentally revealed through the HPLC-MS/MS experiments.

2.3. Bacteria cultivation for nitrile hydratase (NHase), amidase and nitrilase (Nase) enzyme assays and for determining maximum inhibitory concentrations of herbicide for microbial growth

Strains were grown in 10 mL of minimum medium (g L^{-1}: 0.08 $FeCl_3 \times 6H_2O$, 0.05 $FeCl_2$, 0.02 $MgSO_4 \times 7H_2O$, 4.0 Na_2HPO_4, 2.0 KH_2PO_4 and 1.8 glucose), were inducted with 100 µL of Totril® ethanolic solution (stock solution 20 µL/mL) and were incubated for 24 h at 28 °C under orbital stirring (156 rpm). Then, cells were harvested by centrifugation for 10 min at 3000 rpm and at 60 m/s^2. The supernatant was discarded and the biomass was rinsed and resuspended in phosphate buffer (pH 7.2, 10 mM) to reach a final cellular concentration of 200 mg mL^{-1}.

2.4. Nitrile hydratase (NHase), amidase and nitrilase (Nase) enzyme assays

Enzyme assays for nitrile hydratase, amidase and nitrilase were performed according to Angelini et al. (2015).

2.5. Experiments for determining the maximum inhibitory concentration of herbicide for microbial growth

In a 96-well microtiter plate, a serial dilution of aqueous buffered solution (pH 7.2, 10 mM) with 250 mM of Totril® solution was performed to achieve final concentrations of 0.97, 1.95, 3.90, 7.80, 15.6, 31.3, 62.5, 125 and 250 mM in a volume of 100 µL. Then, 100 µL of NB media containing cell suspensions (200 mg mL^{-1}) was added to each well. Microtiter plates were incubated under orbital stirring (181 rpm) at 29 °C. After 24 h, 100 µL of MTT 0.025% was added to each well and the microtiter plate was incubated again for 1 h. Cell viability levels were measured by the visual inspection of the microtiter plate. A color change from yellow to purple denotes the occurrence of mitochondrial respiration and consequently cell viability. All of the assays and microbial control experiments were performed in duplicate.

2.6. Biodegradation experiments

2.6.1. Pre-inoculum preparation

Strains were grown in 1-L Erlenmeyer flasks with 350 mL of NB medium, glucose (10 g L^{-1}) and yeast extract (1 g L^{-1}) at 28 °C under 156 rpm for 24 h.

2.6.2. Enzymatic induction

It were transferred 8% of the pre-inoculum to a 500 mL-Erlenmeyer flask containing 250 mL of induction medium ($NaH_2PO_4 \times H_2O$ 1.03 g L^{-1}, $KHPO_4$ 2.0 g L^{-1}, $MgSO_4 \times 7H_2O$ 0.50 g L^{-1}, $FeSO_4 \times 7H_2O$ 0.03 g L^{-1}, $CaCl_2$ 0.05 g L^{-1}, yeast extract 0.10 g L^{-1}, glucose 10 g L^{-1}, $CoCl_2 \times 6H_2O$ 0.05 g L^{-1} and isobutyronitrile (55 mM solubilized in 2.5 mL ethanol) and we stirred under orbital conditions (156 rpm) at 28 °C for 24 h.

2.6.3. Biodegradation experiments

The resulting biomass from the enzymatic induction was harvested by centrifugation (10 min, 3000 rpm, and 60 m/s^2) and was rinsed and re-suspended in phosphate buffer (100 mM, pH 7.5) to achieve OD_{610} = 2. It was added Totril® to this mixture (0.1 mM solubilized in 4.5 mL of ethanol). The biotransformation reaction was facilitated under

orbital stirring at 28 °C and was monitored by HPLC-MS/MS for 7 days. Microbial and substrate control experiments were performed in parallel.

2.7. HPLC-MS/MS analysis

Analyses were performed using a AB SCIEX 3200 QTRAP HPLC-MS/MS and the analytes were separated on a Kinetex C18 (150 mm × 4.6 mm, 5 μm particle size) at 40 °C. Gradient elution was applied in binary solvent system water: acetonitrile: 0.0–0.5 min (10% acetonitrile), 0.5–14.0 min (100% acetonitrile), 14.0–17.0 (100% acetonitrile), 17.0–17.5 (10% acetonitrile), and 17.5–22.0 min (10% acetonitrile). The flow rate was set to 1000 μL min^{-1} and aliquots diluted in methanol were drawn through biodegradation experiments (20 μL): water (1000 :1).

Electrospray ionization (turbo ion spray source) was applied in the negative mode (ESI-(-)) for all of the compounds, and a mass analysis was performed to scan ions in the enhanced mode (EMS, enhanced mass scan) using a linear ion trap analyzer. The ion source parameters were as follows: ion spray 4500 V, temperature 550 °C, gas 1 45 psi, gas 2 45 psi, curtain gas 12 psi, declustering potential 40 V, and entrance potential 11 V. Ion fragment experiments (EPI, enhanced product) were also performed using a liner ion trap to obtain more structural information. Collision energy levels ranged from 25 to 50 V.

3. Results and discussion

3.1. Screening of bacterial degraders

Indigenous bacteria were isolated from the enrichment cultures of soil currently exposed to Totril®, a commercial formulation of ioxynil octanoate formulated by the Bayer company. The enrichment culture yielded 29 bacterial isolates of the following codes: MLH-29 to MLH-40, MLH-42 to MLH-45, MLH-48, MLH-51 to MLH-57, and MLH-59 to MLH-63. The first screening evaluated the presence of enzymes of nitrile metabolism through a colorimetric assay (Angelini et al., 2015). This was found because nitrile hydratase (EC 4.2.84.1), amidase (EC 5.5.1.4) and nitrilase (EC 3.5.5.1) are well known for taking part in benzonitrile herbicide biodegradation, yielding the respective amides and/or carboxylic acids from nitrile (Scheme 1). Among them, 11 strains (MLH- 31, 34, 36, 42, 43, 54, 55, 59, 60, 61 and 63) were selected.

The next step involved determining the maximum concentration of ioxynil octanoate that did not inhibit microbial growth. A MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to evaluate cell viability levels against increasing herbicide concentrations (0.97, 1.95, 3.90, 7.80, 15.6, 31.3, 62.5, 125 and 250 mM). Cell viability levels were measured by visual inspecting the microtiter plate. Color changes from yellow tetrazole (MTT) to purple formazan denote mitochondrial respiration and thus cell viability (Table 2, Supplementary Material). From this MTT-assay, strains MLH-31 and MLH-61 were selected for the biodegradation experiments. A concentration of 15.6 mM was found to be 31 times higher than ioxynil octanoate concentrations found in the soil that the microorganisms were isolated from. The two strains were identified by Sanger sequencing: MLH-31 presented 100% similarity with *Lysinibacillus boronitolerans* while MLH-61 presented 99% similarity with *Bacillus cereus*.

3.2. Biodegradation experiments and HPLC-MS/MS monitoring

Biodegradation products were monitored, identified and characterized by HPLC-MS/MS analysis. Tandem mass spectrometry ESI-MS (/MS) has been established as the main approach to reaction monitoring. The technique is used to with sensitivity and gentleness “fish out” ionic and ionized intermediates directly from reaction solutions into the gas phase, in which proper characterization using a variety of MS techniques can be performed (Vaz et al., 2013). A batch-resting cells experiment in phosphate buffer (pH 7.2, 100 mM) and Totril® (1.0 mM) with pre-induced strains of *Lysinibacillus boronitolerans* MLH-31 and *Bacillus cereus* MLH-61 (OD_{600} = 2.0) at 28 °C under orbital stirring (156 rpm) protected from light was performed to evaluate herbicide biodegradation. Each sample was centrifuged, supernatant was diluted in methanol: water solution (1000: 1) and it was directly analyzed by HPLC-MS/MS. In parallel, substrate stability and microbial control experiments were performed. It is well known that ioxynil octanoate easily undergoes ester hydrolysis in alkaline medium and that its C-halogen bond can be photohydrolyzed in aqueous solution (Holtze et al., 2008; Malouki et al., 2004). Our substrate stability experiments show that no abiotic degradation or phototransformation occurred under the experimental conditions used. In our microbial control experiments, no ioxynil octanoate biodegradation products or metabolites were observed.

The chromatographic method was applied to a sample of Totril®. The chromatogram revealed the presence of active ingredient ioxynil octanoate (MW 497 g mol^{-1}; retention time 15.15 min) and of minor concentrations of ioxynil (MW 371 g mol^{-1}; retention time 7.23 min) besides hydrocarbon solvents. Standard commercial samples of ioxynil and ioxynil octanoate were also analyzed to confirm compound retention times and fragmentation patterns. An HPLC-ESI-(-)-MS analysis of both ioxynil and ioxynil octanoate standard samples revealed the presence of ions of *m/z* 370 corresponding to deprotonated species $[M - H]^-$ and $[M - C_8H_{16}O]^-$, respectively, as is shown in Fig. 1. These results denote the presence of an ester cleavage in the ion source.

Ester cleavage in the mass spectrometer ion source and enzyme hydrolytic activity were studied using HPLC peak areas drawn from the substrate control and biodegradation experiments. It was assumed that the decrease in the chromatographic peak area of ion *m/z* 370 at 15.15 min identified from the biodegradation experiments denoted the presence of hydrolases responsible for enzymatic ester cleavage.

During biodegradation monitoring, four major ions of *m/z* 263, 345, 388 and 389 were easily and directly intercepted and further characterized via ESI-(-)-MS/MS (Fig. 2). Ions of *m/z* 388 and 389 were characterized as deprotonated ioxynil amide and carboxylic acid, respectively. It was found that the ion of *m/z* 345 corresponds to the deprotonated product of the $C_{aromatic}$-$C_{carboxylic\ acid}$ cleavage while the ion of *m/z* 263 corresponds to deprotonated mono-deiodinated carboxylic acid.

Kinetics of the biodegradation of ioxynil octanoate by *Lysinibacillus boronitolerans* MLH-31 are shown in Fig. 3. After 24 h, the product of ester hydrolysis appears as a primary metabolite (ion of *m/z* 370) followed by the correspondent amide (ion of *m/z* 388). These results denote the presence of enzymes hydrolase and nitrile hydratase, respectively, during the first phase of ioxynil octanoate biodegradation. The ion of *m/z* 370 reached maximum intensity levels within 48 h and then decreased for up to 144 h, after which it was no longer observed. No accumulation of amide occurred (ion of *m/z* 388) throughout the process, indicating that this metabolite was continuously biodegraded by an amidase, leading to the generation of the corresponding carboxylic acid (ion of *m/z* 389). After 48 h, the ion *m/z* 389 appeared and expanded at a higher rate than the others, and likely due to the simultaneous actions of two enzymes that can lead to the formation of carboxylic acid: a nitrilase that uses the ioxynil as a substrate and the amidase which uses the recently and continuously formed amide. Between 48 and 72 h, *m/z* 263 ions (mono-deiodinated product) appear, denoting the presence of a dehalogenase. The last metabolite is observed after 48 h and corresponds to the ion of *m/z* 345 originating from the action of carbon-carbon lyase responsible for the cleavage of a $C_{aromatic}$-$C_{carboxylic\ acid}$ bond, suggesting that the main substrate of this enzyme is carboxylic acid. After 168 h, none of the mentioned metabolites were observed any longer.

Based on the HPLC-MS/MS results, we propose the ioxynil octanoate biodegradation pathways shown in Fig. 4.

According to the literature, ioxynil was biodegraded to the corresponding carboxylic acid at a rate of 40% after 168 h (7 days) by *Aminobacter* MSH1 (OD_{600} ~ 1) while *Rhodococcus rhodochrous* PA-34, *Rhodococcus* sp. NDB 1165 and *Nocardia globerula* NHB-2 (OD_{600} ~ 2 for all three strains) degraded ioxynil into its correspondent carboxylic acid at a rate of 60% over 20 h, and no further degradation was observed over 3 days. Through a batch-resting cell experiment, a rate of 97% ioxynil octanoate biodegradation by the bacteria *Lysinibacillus boronitolerans* MLH-31 (OD_{600} ~ 2), an indigenous strain isolated from ioxynil octanoate-exposed soil, was achieved over 168 h (7 days). The *Bacillus cereus* MLH-61 (OD_{600} ~ 2) strain degraded 75% of the ioxynil octanoate within 7 days through a similar pattern of enzymatic degradation though distinct kinetic behaviors (data not shown).

4. Conclusion

This paper sheds light on the biodegradation pathways of ioxynil octanoate by Gram-positive bacteria *Lysinibacillus boronitolerans* MLH-31. This investigation suggests that hydrolases are the first enzymes acting in the enzymatic cascade, yielding the active ingredient ioxynil. Nitrile hydratases then convert it to the less toxic amide, the substrate of amidase which immediately hydrolyzes it into the corresponding carboxylic acid. Carboxylic acid can also be directly obtained from nitrilases. Moreover, this carboxylic acid suffers from reductive monodeiodination catalyzed by dehalogenases and a simultaneous $C_{aromatic}$-$C_{carboxylic\ acid}$ bond cleavage catalyzed by lyases. This is the first report to elaborate on *Lysinibacillus boronitolerans* bacteria as an ioxynil octanoate degrader. The two new observed biodegradation metabolites (3-iodo-4-hydroxybenzoic acid and 1,3-diiodophenol) were also included in the herbicide pathway. In addition, *Lysinibacillus boronitolerans* can biodegrade ioxynil octanoate over a shorter period than other bacteria described in the literature, revealing its potential as a benzonitrile herbicide degrader. Experiments with pilot plants simulating natural conditions should be done to explore the behavior of ioxynil octanoate degradation under natural field conditions by this microorganism.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bcab.2018.01.002.

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