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ORIGINAL ARTICLE

Batch studies on the biodegradation of paracetamol and 1,4-hydroquinone by novel bacterial strains isolated from extreme environmental samples and the identification of candidate catabolic genes

Alba Lara-Moreno[1,2] · Fatma El-Sayed[3] · Cymon J. Cox[1] · Maria C. Costa[1,3] · Jorge D. Carlier[1]

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Abstract
The emerging pollutant paracetamol (APAP) is one of the most prescribed drugs worldwide. In addition, APAP and its main metabolites, namely, 4-aminophenol (4-AP), hydroquinone (H2Q), benzoquinone (BQ), and 2,5-dihydroxy-1,4-benzoquinone (2,5-OH-BQ), among others, are frequently detected in wastewater treatment plants (WWTPs) influents, effluents, and the environment. Thus, continuous release into the environment, especially aquatic environments, is a source of general concern. Six APAP-degrading bacterial strains were isolated from two mine samples from the Iberian Pyrite Belt (Lousal and Poderosa mines). *Mycolicibacterium aubagnense* HPB1.1, which was isolated using enrichment cultures from the Poderosa mine sample in the presence of H2Q as the sole carbon source, also showed APAP biodegrading capabilities. Pure cultures of this strain degraded 34.3 mg L^{-1} of APAP in 5 days and 9.4 mg L^{-1} of H2Q in 4 days. Interestingly, BQ and 2,5-OH-BQ were detected as metabolites resulting from H2Q abiotic degradation, but these compounds were removed in the strain's cultures. Furthermore, *M. aubagnense* HPB1.1 whole-genome was sequenced, and its encoded proteins were aligned with enzymes of APAP-degrading bacteria recovered from databases and literature aiming to identify candidate catabolic genes. Putative amidases, deaminases, hydroxylases, and dioxygenases, responsible for the degradation of APAP by the HPB1.1 strain, were identified by similarity, corroborating its ability to transform APAP and its intermediate metabolite H2Q into less toxic metabolic compounds due to their capacity to break the aromatic ring of these molecules.

Keywords Bioremediation · Aerobic biodegradation · Wastewater treatment plants · *Mycolicibacterium aubagnense* HPB1.1 · Emerging contaminant · Biodegrading genes

Introduction

Over the last two decades, environmental concerns have increased beyond the awareness of traditional pollutants such as polychlorinated biphenyls, dioxins, organochlorine, and organophosphorus pesticides. These concerns have extended to emerging pollutants introduced into the environment mainly through anthropogenic activities (Ternes et al. 2004; Kock et al. 2023). The true threat behind these emerging pollutants is that their environmental and human toxicological effects have yet to be fully evaluated (Rosenfeld and Feng 2011). Pharmaceuticals such as endocrine-disrupting drugs, analgesics, antibiotics, hormones, anti-inflammatory, antidiabetic, and antiepileptic drugs have been considered emerging pollutants because many studies have detected their presence in different matrices (surface water, groundwater, or soil) (Mohapatra et al. 2021). Pharmaceuticals are primarily introduced into the environment through continuous effluent release from pharmaceutical industries, human and veterinary health facilities, and particularly, wastewater

✉ Alba Lara-Moreno
alara9@us.es

Jorge D. Carlier
jcarlier@ualg.pt

[1] Centre of Marine Sciences (CCMAR) , University of the Algarve, Gambelas Campus, 8005-139 Faro, Portugal

[2] Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Seville, 41012 Seville, Spain

[3] Faculty of Sciences and Technologies , University of the Algarve, Gambelas Campus, 8005-139 Faro, Portugal

treatment plants (WWTPs). This is considered unavoidable due to population growth and aging, which means that these compounds will be increasingly present in urban wastewater (Silva et al. 2021).

Paracetamol, also known as N-acetyl-p-aminophenol and frequently shortened as APAP, is one of the most widely used non-prescribed analgesic, and antipyretic drugs (Żur et al. 2018; Shabani et al. 2021). However, APAP and its degradation products 1,4-hydroquinone (H2Q) and 4-aminophenol (4-AP) are considered not only emerging pollutants but also micropollutants detected in the environment at concentrations ranging from below ng L^{-1} to µg L^{-1} (Kim and Zoh 2016). Despite that the level of APAP in sewage effluents and natural waters is considered low (ng L^{-1} to µg L^{-1}), varied negative effects have been reported (Heberer 2002). These include reproductive or DNA damage, accumulation in tissues, oxidative stress, lipid peroxidation, and behavioral changes observed in algae, microcrustaceans, mollusks, or teleost fish (Gómez-Oliván et al. 2014; Minguez et al. 2016; Islas-Flores et al. 2017). Based on Regulation (EC) No 1907/2006, APAP was categorized as harmful to aquatic organisms based on an EC_{50} concentration between 11 and 100 mg L^{-1} and as very toxic to aquatic organisms based on an EC_{50} concentration < 1 mg L^{-1} after long-term exposure. The intermediate product of APAP biodegradation, 4-AP, is considered a dead-end metabolite (Mackie et al. 2013). 4-AP was reported for its considerable nephrotoxicity, mutagenicity, teratogenicity, and DNA cleavage induction in mouse and human lymphoma cells (Wang and Feng 2023). In addition, H2Q, the main metabolite of APAP, is widespread in the environment since it is usually produced from the advanced oxidation of several aromatic compounds (as APAP) and also due to its extensive use in various activities such as photography, dye intermediates, stabilizers in paints, varnish oils, and motor fuels. However, H2Q has proven to be more toxic and less degradable than phenol (Santos et al. 2004). Indeed, H2Q is known as a hematotoxic and carcinogenic agent associated with harmful effects in occupational environments. Moreover, in the environment, H2Q has shown increased toxicity for aquatic organisms (Enguita and Leitão, 2013).

Levels of pollutants, including APAP and its metabolites, are rising in WWTPs, which accordingly accumulate in the environment. The conventional decontamination method of chlorination, as well as advanced methods including membrane bioreactors, nanofiltration, reverse osmosis, and carbon nanocomposites with magnetic properties, are not sufficiently tailored for pharmaceutical removal (Kim et al. 2018; Tiwari et al. 2017). Both current and conventional methods mostly convert APAP to its metabolites which are even more toxic (Chopra and Kumar 2020). Therefore, there is still an urgent need to develop strategies aimed at improving the removal of these chemicals.

Bioremediation is a comprehensive strategy that utilizes biological systems, primarily bacteria, fungi, and plants, to clean contaminated environments by oxidizing, immobilizing, or transforming pollutants. Its main objective is to decrease pollution levels to those that are undetectable, nontoxic, or acceptable (i.e., within limits set by regulatory agencies) (Vishwakarma 2020). Microbial biodegradation is mostly considered an effective, sustainable, eco-friendly, and cost-effective approach for the removal of APAP (Vargas-Ordóñez et al. 2023). To boost the effectiveness of degradation, three strategies have been suggested: natural attenuation (using the ability of the intrinsic microbial community to degrade the contaminant), bioaugmentation (the addition of living cells capable of degradation), and biostimulation (the addition of limiting nutrients to support microbial growth) (Lara-Moreno et al. 2022a). Pollutant-degrading microorganisms are obtained using enrichment cultures in the presence of high concentrations of contaminants (Palma et al. 2021; Lara-Moreno et al. 2022b; Vargas-Ordóñez et al. 2023). Several studies have shown improved bioremediation using bioaugmentation in contaminated water by APAP. A varied panel of bacterial strains has been identified during the last two decades for their ability to biodegrade high APAP concentrations (some of them above 50–3000 mg L^{-1}) using it as a sole carbon source, including members of *Pseudomonas* (Poddar et al. 2022; Vargas-Ordóñez et al. 2023; Żur et al. 2018; Hu et al. 2013), *Bacillus* (Chopra and Kumar 2023), *Stenotrophomonas* (Zhang et al. 2013), *Ensifer* (Park and Oh 2020), or *Shinella* (Chen et al. 2022), among others. Compared with the number of APAP biodegrading strains, fewer microbial strains have been proven for their ability to biodegrade *4*-AP and H2Q. For example, Khan et al. (2009) reported that *4*-AP biodegradation was induced by *Pseudomonas* sp. ST-4 at different conditions (pH, temperature, and glucose concentration). In another study, *Moraxella*, *Pseudomonas*, *Sphingomonas*, *Burkholderia*, *Variovorax*, *Azospirillum*, *Brachymonas*, and *Cupriavidus* have been described for their ability to utilize H2Q which may be a product of not only APAP but also other phenolic compounds such as 4-chlorophenol, 4-fluoro-, 4-bromo-, 4-iodo-, and 4-nitrophenol degradation (Enguita and Leitão, 2013). More recently, Zhao (2017) reported that H2Q is removed by the endogenous microbiota of granular sludges. Despite its toxicity, only one biodegradation study (Harbison and Belly 1982) has been reported using H2Q as the initial compound.

In this work, bacterial strains with the ability to degrade APAP and its metabolites as the only source of carbon and energy were isolated using enrichment cultures inoculated with samples from extreme environments of mines from The Iberian Pyrite Belt. The isolate, *Mycolicibacterium aubagnense* HPB1.1 was identified as the most effective degrading bacteria. Furthermore, this species was not previously

reported as being able to remove the studied compounds. Thus, whole-genome sequences of the strain were obtained, and their encoded proteins compared to APAP-degrading enzymes retrieved from databases and literature aiming to identify candidate catabolic genes for future works on gene function analysis to provide new information about the mechanism of APAP biodegradation that lately will be useful for genetic bioaugmentation to improve wastewater treatment.

Materials and methods

Materials

Analytical grade paracetamol (N-(*4*-hydroxyphenyl) acetamide, purity ≥ 99%) and 1,4-Hydroquinone (Benzene-1,4-diol, purity ≥ 99%) were purchased from Sigma-Aldrich, USA.

Five mine samples (LA, LC, PB, PDE, and PF) from the Iberian Pyrite Belt (considered one of the most important metallogenic regions in the world) were used to obtain APAP and H2Q degrading bacteria. LA and LC samples were collected from white and yellow wall sediments of the Lousal mine, respectively (38°02′01.8"N 8°25′32.2"W) located in Grândola (Portugal). The Lousal mine was mostly active from 1900 to 1988, and it was known as an important massive sulfide deposit for fertilizer production. The deposit was primarily mined for pyrite (iron sulfide) with about 50 Mt of ore with 1.4% Zn, 0.8% lead, and 0.7% copper (Relvas et al. 2014). PB, PDE, and PF samples were collected in the Poderosa mine (37°44′54.8"N 6°39′19.9"W) based in Campillo (Huelva, Spain), from wall sediment, wall moonmilk, and sand sediment, respectively. The Poderosa mine was working between 1864 and 1924, and it presents high levels of copper as chalcopyrite, chalcocite, and covellite. Poderosa has been an important source of copper, exploiting more than 600 000 tonnes of copper during its activity period (Grande et al. 2016). X-ray diffraction (XRD) characterization by Palma and Costa (2024) revealed for the Lousal mine's samples a predominant composition of silicates (silicon dioxide, quartz, olivine), phyllosilicates (kaolinite, muscovite), oxides (hematite), carbonates (calcite), sulfide (arsenopyrite, octosulfur), and aluminum phosphate, while for the Poderosa mine's samples it revealed mainly a composition of silicates (quartz), phyllosilicates (muscovite), sulfates (pickeringite, gypsum, natrojarosite, jarosite), and phosphate (brushite).

It is generally accepted that there is a great biotechnological potential in microbial communities from extreme environments, since they have microorganisms that evolved with mechanisms allowing their adaptation to hostile conditions. For example, strains with biotechnological potential have already been isolated from moonmilk, an extreme environment found in caves and mines composed mainly of fine CaCO3 crystals where extremophiles and non-extremophiles are found (Hui et al. 2021; Kosznik-Kwaśnicka et al. 2022).

Methods

Collecting of microbial consortia from mine samples

The samples collected from Lousal (LA and LC) and Poderosa (PB, PDE, and PF) mines were washed with Ringer's solution (Sigma-Aldrich, USA). This washing procedure was performed in 50 mL flasks with 9 mL of Ringer's solution and 1 g of sample with 150 rpm of orbital agitation for 4 h. After that, 500 µL of the washing solution was transferred to a 50 mL flask with 5 mL of Nutrient broth (NB, g L^{-1}: 10.0 peptones; 1.0 beef extract; 2.0 yeast extract; 5.0 NaCl) and incubated at 25 °C for 48 h. After incubation, cultures were preserved in glycerol (20% v/v) stored at − 80 °C.

Enrichment culture of paracetamol and 1,4-hydroquinone degrading consortia

220 µL of microbial suspension from a glycerol stock kept at − 80ºC was inoculated into 20 mL of LB broth in a 100 mL Erlenmeyer flask and incubated at 30 °C under shaking conditions of 160 rpm for 24–48 h. The grown seed cultures of Poderosa mine consortia (PB, PF, and PDE) and Lousal mine consortia (LA and LC) were centrifuged at 4000 rpm for 20 min. After discarding the supernatant, pellets were washed twice by resuspension in 10 mL of Mineral salt medium ((MSM, g L^{-1}): 0.5 K_2HPO_4; 0.5 KH_2PO_4; 1.0 NH_4HPO_4; 0.5 $MgSO_4$; 0.01 NaCl; 0.2 $MgCl_2$; 0.03 $CaCl_2$; 0.00034 $MnSO_4$; 0.00043 $ZnSO_4$; 0.00035 $NH_4(Mo{\cdot}7O_2)$ $4H_2O$; 0.0004 $CoCl_2$; 0.0001 EDTA) followed by centrifugation (4000 rpm for 20 min). The obtained pellet was resuspended in MSM. Enrichment cultures were performed in a 100 mL sterilized Erlenmeyer flask. Each flask contained 45 mL of MSM supplemented with 500 mg L^{-1} of APAP and a volume of microbial inoculum to reach a final $OD_{600} = 1$. The flasks were kept at 30 °C under shaking conditions of 160 rpm, and every 7 d, 10 mL of the culture was transferred to a new flask containing the new sterile MSM with 500 mg L^{-1} of APAP and incubated again. This process was repeated three times. The same proceeding was conducted with H2Q (500 mg L^{-1}) using PB, PF, and PDE. In the case of APAP, at the end of the incubation period planned for each enrichment culture, 1 mL of each culture was centrifuged at 4000 rpm for 20 min, then tenfold dilution was conducted using sterile MSM and the diluted samples were taken for drug monitorization using UV–vis spectrum scans at 245 nm.

Isolation of potential paracetamol and 1,4-hydroquinone degrading bacteria

100 µL of each tenfold dilution from final enrichment cultures were spread on MSM agar plates supplemented with APAP and H2Q separately at a concentration of 500 mg L^{-1} as the sole carbon source added; hence, bacteria that grow on the plates are considered potentially drug biodegrading. After confirming the microbial growth, biomass was spread on LB-drug plates to obtain morphologically distinct colonies. During one week of incubation at 30 °C, the plates were checked daily, and morphologically distinct colonies were picked separately and streaked on MSM-drug (500 mg L^{-1}) plates to confirm the isolate's ability to utilize the drug as a sole carbon source. To prevent bacterial growth in the solid medium due to impurities from the agar that could serve as carbon sources, we have used Ultrapure Noble Agar (Thermo Scientific, Ref. J10907, LOT: 213,191). Pure colonies of each isolate were suspended into the cryopreservation medium in a cryovial for long-term storage at − 80 °C.

16S rRNA gene sequencing and taxonomic classification

Genomic DNA was extracted from a pure culture of each of the 15 potential isolates that were able to utilize APAP or H2Q as the sole carbon source, using NZY microbial gDNA isolation kit (Nzytech, Portugal). 1 mL of each culture was centrifuged (4000 rpm for 20 min) and DNA was extracted from cell pellets as described in the kit. The 16S rRNA gene was amplified using a Supreme NZYTaq 2 × Green Master Mix (Nzytech, Portugal) with universal primers for prokaryotes: the universal primers 8F (also known as fD1) (5′- AGA GTT TGATCC TGG CTC AG -3′) (Weisburg et al. 1991) and 1492R (5′-GGT TAC CTT GTTACG ACT T-3 ′) (Lane 1991). The PCR amplification was confirmed by gel electrophoresis using ENDUROTM electrophoresis system (Labnet, USA), which was carried out in 1% (w/v) agarose gel in 1 × TAE buffer. The amplified products were sequenced by the Sanger method in both directions (forward and reverse), with the same primers that were used for PCR amplification. A capillary electrophoresis sequencing system (Genetic Analyzer, Model 3130xl, Applied Biosystems, Foster City, USA) was used for Sanger sequencing. The taxonomic classification was based on the results obtained by nucleotide alignment with the "16S ribosomal RNA sequences (bacteria and archaea)" database, using the BLASTn (Basic Local Alignment Search Tool nucleotide) tool (Altschul et al. 1990) at the NCBI Web BLAST site (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Inoculum preparation

An aliquot of bacterial isolate culture was transferred to 5 mL of LB broth supplemented with 500 mg L^{-1} of APAP or H2Q. Incubation proceeded for 24 or 48 h at 25 °C under shaking conditions of 160 rpm. 5 mL of each grown culture was inoculated into two separate 100 mL aliquots of LB-drug (500 mg L^{-1}) medium dispended in 200 mL bottles and incubated under the same conditions. Microorganisms were harvested and centrifuged after 24–48 h and subsequently washed twice using MSM, to remove any possible LB residues, before initiation of the experiments.

Paracetamol biodegradation assay in solution

APAP biodegradation experiments were performed in duplicates in 200 mL sterilized glass bottles containing 60 mL of MSM supplemented with APAP or H2Q at a concentration of 50 mg L^{-1}. Bacterial inoculum was added in the volume necessary to adjust initial OD_{600} ~ 1 (10^8 CFU mL^{-1} approximately). Glass bottles were kept shaking (160 rpm) at 30 °C in the dark. Samples were taken at the initial time and at different incubation times (0, 5, 10, 15, 19, and 28 days). Negative controls of uninoculated media were also tested. At each time interval, 1 mL of each test was centrifuged at 4000 rpm for 20 min and the supernatant was filtered using polyether sulfone syringe filters (0.22 µm pore size) for the subsequent HPLC analysis. HPLC conditions were applied as described by Palma et al. (2018) to quantify APAP. A reversed-phase Surf Extreme C18 column (250 mm × 4.6 mm, 5 µm, functional group: a 100 Å pore-sized silica), purchased from ImChem, was used for separation. An Xbridge-C18 guard column (4.6 × 20 mm, 5 µm) purchased from Waters was used at the entry of the separation column. Smartline HPLC set (KNAUER, Germany), with UV-detector 2600 was used and peaks were analyzed using Chromatography-Clarity SW software. The mobile phase consisted of phosphate buffer/methanol (80/20, v/v). The phosphate buffer solution (pH 4.6) was prepared by dissolving 4.5 g KH_2PO_4 and 0.0314 g $K_2HPO_4 \cdot 3H_2O$ in 500 mL of ultrapure water, using phosphoric acid (85%) to adjust pH if necessary. The isocratic mode was applied with 1 mL min^{-1} flow rate. The column was maintained at room temperature. The total run time was 10 min for APAP and the injection volume was 20 µL. The detection was at 245 nm. The samples were quantified by external linear calibration, using six concentrations from 50 to 2.5 mg L^{-1}. The obtained calibration curve had an R^2 of 0.998 and an estimated Limit of Detection (LOD) of 1.6 mg L^{-1}.

1,4-hydroquinone biodegradation assay in solution

H2Q biodegradation in solution was studied in three replicates, conducted in 200 mL sterilized Erlenmeyers flask with 60 mL of MSM, spiked with 10 mg L^{-1} of H2Q as the sole carbon source. The flasks were inoculated with the volume of bacterial inoculum (HPB1.1 strain) necessary to adjust the initial OD_{600} = 0.8–1. In the case of negative control, the flasks were not inoculated with the bacterium. Then, the Erlenmeyers flasks were kept at 30 °C and shaken (125 rpm) in the dark for 7 days. During this period samples were taken at the initial time and after 2, 4, 6, and 7 days. Before measuring the samples in the HPLC, they were processed in the same way as mentioned in the sect Inoculum preparation.

The concentration of H2Q in the samples was quantified using an Ultra High Performance Liquid Chromatography Nexera system (SHIMADZU), equipped with an XBridgeTM C18 5 µm, 4.6 × 250 mm column (Waters, USA). For the H2Q samples, the mobile phase composition was Methanol:Water (10:90 v/v), adjusted to a pH ~ 3 with orthophosphoric acid 85%, using an isocratic method. The flow rate was set at 1.3 mL min^{-1}, with an injection volume of 200 µL. The total run time was 8 min. The detection wavelength was at 190 nm for H2Q and 240 nm for its metabolites (2,5-OH-*1,4*-BQ and 1,4-benzoquinone). The retention time for the samples was approximately 4 min for H2Q, 4.9 and 5.4 min for 2,5-OH-*1,4*-BQ, and 6.8 min for 1,4-benzoquinone. The samples were quantified by external linear calibration, using five concentrations from 25 to 1 mg L^{-1}. In this case, the calibration curve had an R^2 of 0.9992 and an estimated LOD of 0.5 mg/L^{-1}.

1,4-hydroquinone adsorption study on bacterial biomass

After the H2Q biodegradation test in solution, an extraction assay in triplicates was conducted to recover the concentration of H2Q accumulated in bacterial cells. The supernatant (42 mL) and the bacterial biomass were centrifugated (20 min, 4000 rpm). After centrifugation, the supernatant was separated from the bacterial pellet. The solvents used for the solid–liquid extraction were selected based on Li et al. (2006). For this, 4.2 mL of Methanol + 0.1% of acetic acid (pH = 4) were added to the Falcon tubes containing the bacterial cells. To disrupt the cells, the extraction procedure involved 20 s vortex mixing, followed by 5 min of sonication and 10 min of centrifugation at 4000 rpm. The quantification of the recovered H2Q from the cells was performed using HPLC, as described in Sect. "Methods".

Genome sequencing data and gene annotation of *Mycolicibacterium aubagnense* HPB1.1.

M. aubagnense HPB1.1 was inoculated in 5 mL of LB liquid medium and incubated at 30 °C at 150 rpm for 24 h. Afterward, an aliquot was centrifugated at 10,000 rpm for 3 min. The genomic DNA was extracted from the collected cells using NZY microbial gDNA isolation kit (Nzytech, Portugal). The DNA concentration was quantified by an ultra-trace UV spectrophotometer (Nanodrop 1000, Thermo Scientific). The genome of *M. aubagnense* HPB1.1 was sequenced to obtain useful genetic information about the genes involved in the APAP biodegradation pathway. The HPB1.1 genome was sequenced by Integrated Microbioma Resource (IMR, Dalhousie University, Canada) using PacBio Sequel at coverage from 33 × to 203x (https://imr.bio). The generated sequencing reads were assembled using PacBio's SMRTlink genome assembler version 10. The bacterial genome has been annotated using the RAST tool (Rapid Annotation Subsystem Technology) (Aziz et al. 2018) and NCBI Prokaryotic Genome Annotation Pipeline. The sequencing data generated in this study were deposited in the National Center for Biotechnology Information (NCBI), under the BioProject ID PRJNA955654 and BioSample ID SAMN34178714. The whole genome of *M. aubagnense* HPB1.1 has been deposited in DDBJ/ENA/GenBank under accession numbers CP122994-CP12299.

In silico analysis: nucleotide and protein sequence analysis

First, a set of reference protein sequences involved in the APAP biodegradation pathway was recovered from databases and literature as explained in more detail in the Results and Discussion section, namely in sub Sect."In silico prediction of genes involved in the paracetamol degradation pathway". Afterward, each reference protein was used as a Query in alignment searches to the genome of *M. aubagnense* HPB1.1 using the NCBI online search tool tblastn, a variant of the BLAST algorithm that compares a protein sequence against a nucleotide sequence database, where the nucleotide sequences are dynamically translated into protein sequences in all six possible reading frames during the search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The algorithm parameters used were those set by default (Max target sequences: 100, Expect threshold: 0.05, Word size: 5, Max matches in a query range: 0, Matrix: BLOSUM62, Gap costs: Existence: 11 Extension: 1, Compositional adjustments: conditional compositional score matrix adjustment, Filter: low complexity regions). Finally, the genomic annotation data of the hits obtained by this tblastn search were used to get the complete sequences of the found proteins (hits) from the genome annotation created with RAST.

The decision on the identification of a candidate catabolic gene was first based on evaluating the alignment percentage of coverage and the percentage of identity. Then, in the case of various sub-unites of the same enzyme, the consecutive genomic position was considered. Moreover, a study of the catalytic domains of the selected proteins was carried out using Conserved Domain Database (CDD) (https://www.ncbi.nlm.nih.gov/cdd) and InterProScan (Jones et al. 2014; Paysan-Lafosse et al. 2022).

Results and discussion

Screening/Enrichment of the microbial consortia for paracetamol and 1,4-Hydroquinone removal

Two microbial consortia (LA and LC) were obtained from the Lousal mine, while three microbial consortia (PB, PDE, and PF) were obtained from the Poderosa mine. The obtained consortia were enriched in MSM medium with APAP as the sole carbon source. During the incubation period of each culture, a deep brown coloration of MSM-APAP was observed for all enrichment cultures of all microbial consortia except for the LC consortium and the negative control (Fig. 1S). Such medium coloration is associated with APAP biodegradation according to De Gusseme et al. (2011) who reported a brown coloration of the synthetic mineral medium during the microbial degradation of APAP by two described isolates, due to the formation, accumulation and polymerization of degradation intermediates, such as catechol and its derivatives (H2Q and *1,4*-benzoquinone). In another work, Korotaev et al. (2016) studied the brown precipitate formed in the process of APAP biotransformation in the presence of *Rhodococcus Ruber* IEGM 77, concluding that 2,5-diaminobenzene-1,4-diol, 4,6-diaminobenzene-1,3-diol, 2,5-dihydroxy-1,4-benzoquinonediimine and 2-amino-5-hydroxy -1,4-benzoquinoneimine were identified as the structural units of the copolymer responsible of coloration. In summary, the brown precipitate is a consequence of oxidative polycondensation of APAP and 4-aminophenol.

Figure 1 shows a preliminary study to quantify the APAP removal values according to UV–vis molecular spectroscopy analysis to have an idea about the capacity of the initial consortia to degrade APAP, before proceeding to strains isolation and biodegradation studies with pure cultures. The first enrichment culture of consortium LA from Lousal mine after 7 days, showed only 6% APAP removal. On the other hand, the second and third enrichment cultures of consortium LA, which were 14 and 21 days old respectively, showed maximal APAP removal capacity as inferred by APAP removal of 99.7% and 84.1%, respectively. The higher extent of APAP removal reported for the second (14 days) and third (21 days) enrichment cultures compared with the first enrichment culture can be explained based on the enhanced growth of APAP-utilizing strains, favoring the development of bacteria that can use APAP as a source of carbon and energy (Parajuli et al. 2017). The enrichment cultures of Poderosa mine consortia PDE and PB showed values of APAP removal of almost 22%, and in the case of LC and PF consortia removals of 15% were achieved.

Furthermore, the PB, PDE, and PF consortia were used aiming to obtain enriched consortia able to degrade H2Q. In this case, deep brown coloration was observed for Poderosa mine enrichment cultures, but also in the negative control medium (Fig. 2S). This indicates that H2Q was changing due to abiotic factors. It is known that oxygen can act as

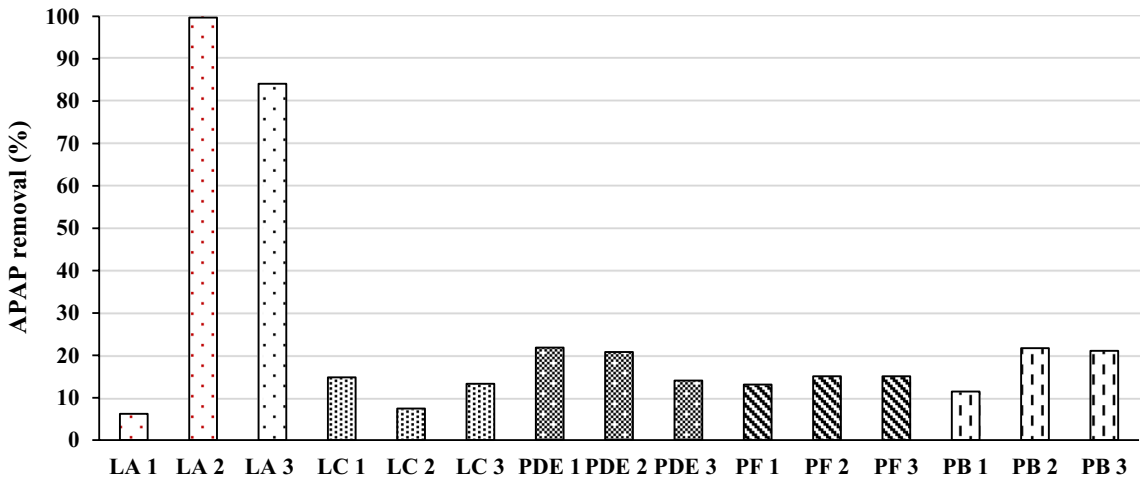


Fig. 1 Paracetamol (APAP) removal in MSM-APAP (500 mg L^{-1}) enrichment cultures of PDE, PB, and PF microbial consortia from Poderosa mine and LA and LC microbial consortia from Lousal mine using UV–vis absorbance (245 nm). 1, 2, and 3 means: 7-day 1st enrichment culture, 14-day-old 2nd enrichment culture, and 21-day-old 3rd enrichment cultures, respectively

an oxidant in the oxidative polymerization of H2Q, leading to the formation of brown polymers. Zhang et al. (2012a) associate brown precipitate with the formation of poly hydroquinone (PH2Q), a redox-active polymer with quinone/H2Q redox-active units in the main chain. Dang et al. (2014) studied something similar and confirmed that H2Q could self-polymerize to form brown polymers based on air oxidation, which is known as PH2Q. Therefore, since there was abiotic transformation of H2Q it was difficult to evaluate if biodegradation occurred or not. Thus, H2Q was not monitored in the enrichment cultures, and all were used to inoculate selective MSM plates aiming to isolate potential H2Q degrading strains.

Isolation and identification of paracetamol and H2Q degrading bacterial strains

Specific potential APAP-degrading strains forming isolated colonies were selected from the solid MSM containing APAP as the only added source of carbon and energy. Fifteen strains were isolated from PB, PDE, PF, LA, and LC consortia. Isolated strains were labeled with the letters P or H (isolated in the presence of APAP or H2Q, respectively) followed by the name of the original consortium (PDE, PB, LC, or LA) plus the enrichment step (2 or 3 for the second or third culture, respectively) and a number from 1 to 4 (PLA2.1, PLA2.2, PLC2.1, PLC2.2, PPDE2.1, PPDE3.1, PPDE3.2, PPDE3.3, PLA3.2, PLA2.3, PLA2.4, PLA3.1, PLC2.3, PLA3.3, PDE3.4). Concerning their taxonomic classification, the NCBI Blast analyses revealed phylogenetic affiliations with identity values of the 16S rRNA sequences with their closest neighbors above 98.99%. However, the sequences presented very high similarities (> 97%) with other close neighbors of the same genus but assigned to different species. Thus, the BLAST alignment was not significant enough to classify the studied isolates at the species level (Yarza et al. 2014).

In what concerns H2Q, only one colony was formed and isolated from the plates inoculated with the enrichment cultures, namely from the plate inoculated with the first enrichment of the Poderosa mine microbial consortium PDE; thus, only one strain showed potential ability to use H2Q as a carbon source. The 16S rRNA gene sequence showed a match of 100% to species from *Mycolicibacterium aubagnense* in the NCBI GenkBank "nt" sequence repository, and our strain was named *M. aubagnense* HPB1.1 based on the same criteria mentioned above. Members of *Mycolicibacterium* were previously included in genus *Mycobacterium* but were separated in a different genus by Gupta et al. in 2018. Table 1 shows the classification of potential APAP and H2Q biodegrading bacterial isolates using 16S rRNA gene sequence analysis.

Paracetamol biodegradation in solution by pure cultures of potentially degrading bacteria

For space and time management reasons, it was decided to randomly choose one representative of each genus for subsequent biodegradation tests with pure cultures in a liquid medium. Thus, six of the fifteen isolates with potential APAP degrading capacity were selected. In addition, the

Table 1 Phylogenetic affiliations of the bacterial strains isolated from Poderosa and Lousal consortia

Strain	Genus	Range of similarity (%)	Phylum/Class, family, genus
PLA2.1	*Bacillus* sp.	99.86–100	Bacillota/Bacilli, Bacillaceae, *Bacillus*
PLA2.2			
PLC2.1			
PLC2.2			
PPDE2.1			
PPDE3.1			
PPDE3.2			
PPDE3.3	*Rhizobium* sp.	99.5–99.8	Pseudomonadota/Alphaproteobacteria, Rhizobiaceae, *Rhizobium*
PLA3.2	*Variovorax* sp.	99.62–99.85	Pseudomonadota/Betaproteobacteria, Comamonadaceae, *Variovorax*
PLA2.3	*Aeromonas* sp.	99.88	Pseudomonadota/Gammaproteobacteria, Aeromonadaceae, *Aeromonas*
PLA2.4			
PLA3.1			
PLC2.3	*Niallia* sp.	97.55–99.19	Bacillota/Bacilli, Bacillaceae, *Niallia*
PLA3.3	*Paraburkholderia* sp.	98.5–100	Pseudomonadota/Betaproteobacteria, Burkholderiaceae, *Paraburkholderia*
PDE3.4			
HPB1.1*	*Mycolicibacterium aubagnense*	100	Actinomycetota/Actinomycetia, Mycobacteriaceae, *Mycolicibacterium*

HPB1.1 is classified to the species level based on its genome sequence (described later in the manuscript)

strain isolated in H2Q selective medium was also selected for APAP biodegradation tests.

Thus, seven strains isolated using enrichment cultures from mine samples were selected to conduct APAP biodegradation assays in liquid MSM supplemented with 50 mg L^{-1} of APAP: *Niallia* sp. PLC2.3, *Bacillus* sp. PPDE3.1, *Rhizobium* sp. PPDE3.3, *Aeromonas* sp. PLA3.1, *Variovorax* sp. PLA3.2, *Paraburkholdeira* sp. PLA3.3, *Mycolicibacterium aubagnense* HPB1.1 (Fig. 2). After 28 days of the biodegradation assay, 1.1 ± 0.1 mg L^{-1} (degraded concentration ± Mean Deviation) of APAP was degraded in the control test, indicating **minimum** abiotic degradation as expected (Zhang et al. 2012a; Dang et al. 2014). *Niallia* sp. PLC2.3, *Aeromonas* sp. PLA3.1 and *Variovorax* sp. PLA3.2 achieved the lowest biodegradation (13.9 ± 0.8, 18.8 ± 3.5 and 18.1 ± 7.9 mg L^{-1}, respectively after 28 days). For *Bacillus* sp. PPDE3.1, a high biotransformation was observed, reaching a degradation of about 23.3 ± 5.2 mg L^{-1} of APAP after 28 days of the assay. *Rhizobium* sp. PPDE3.3 and *Paraburkholderia* sp. PLA3.3 seemed the most promising for APAP removal where APAP removal was 31.9 ± 2.5 mg L^{-1} and 31.4 ± 0.6 mg L^{-1}, respectively. Interestingly, the Poderosa mine *M. aubagnense* strain HPB1.1 selected as potential H2Q degrading also showed APAP removal. As shown in Fig. 2, after 5 days 34.3 ± 0.8 mg L^{-1} APAP was removed, and after 28 days, APAP removal was 46.6 ± 0.1 mg L^{-1}, in the MSM cultures initially with 50 mg L^{-1} APAP. The negative control did not indicate abiotic degradation and high removal was observed in all tests, suggesting the biodegrading capacity of all isolates.

Several authors have reported the ability of some of the studied genera to degrade organic pollutants. Bako et al. (2021) highlighted polychlorinated biphenyl degradation in bioreactors by *Paraburkholderia*. *Rhizobium* sp. has been used for phenol compound degradation (Wei et al. 2008). In the study of Patel et al. (2022), *Niallia circulans* has been reported to degrade azo dye Reactive Red 152. Also, *Mycolicibacterium* sp. has been stated as polychlorinated biphenyls degrading (Steliga et al. 2020) and Naloka et al. (2021) indicated *Mycolicibacterium* sp. as a part of an artificial consortium to degrade fuel oil. However, genera *Paraburkholderia*, *Rhizobium*, *Niallia*, and *Mycolicibacterium* members have not previously been reported as pharmaceuticals-degrading bacteria, including APAP and H2Q. Therefore, more studies should be directed toward further exploring the diversity of potential biodegrading bacteria from mines as representatives of extreme environments. Nevertheless, species of the genera *Aeromonas*, *Bacillus,* and *Variovorax* have been described as drug degraders. *Aeromonas* sp. was able to remove gliazide in MSM supplemented with 0.5 g L^{-1} of gliazide (Ouartsi et al. 2019). *B. thuringiensis* B1 degraded 20 mg L^{-1} of ibuprofen in MSM supplemented with 1 mg L^{-1} of glucose (Marchlewicz et al. 2017). Furthermore, *Bacillus subtilis* and *Bacillus cereus*

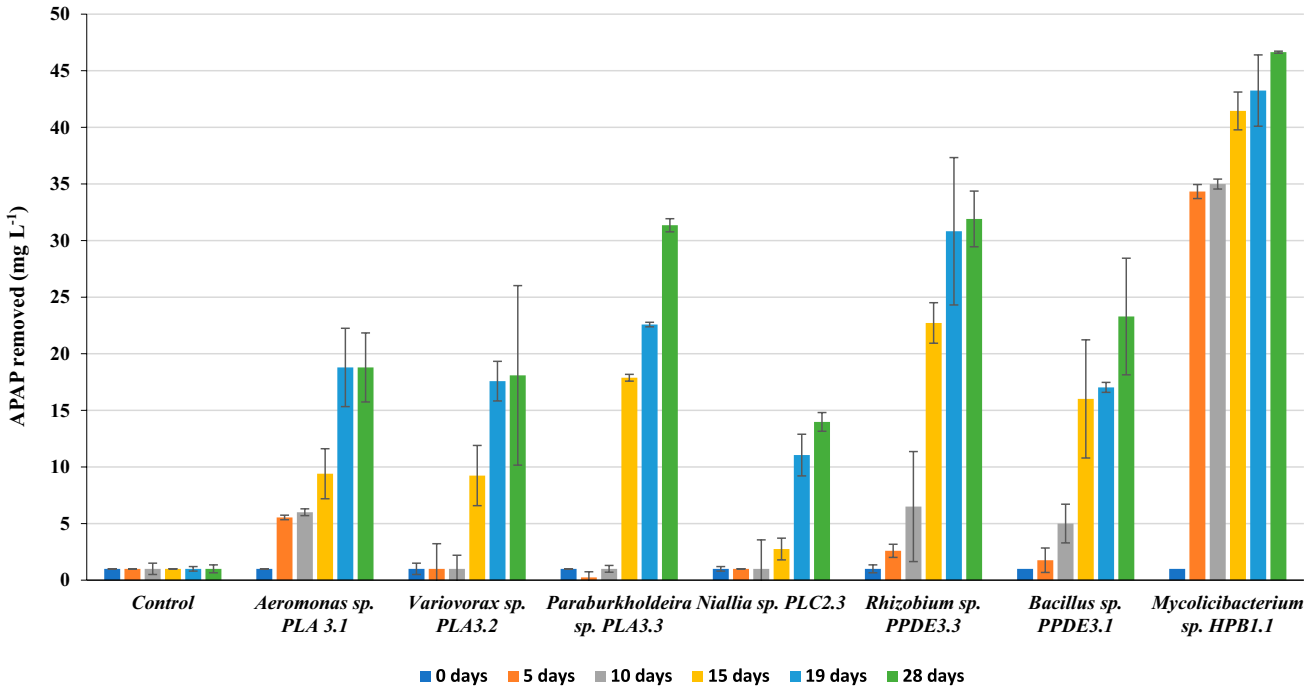


Fig. 2 Paracetamol (APAP) removal in MSM-APAP (50 mg L^{-1}) (Control) and after inoculation of bacterial isolates (Tests) during the biodegradation experiments. [Removed] = [initial]-[after incubation], based on HPLC analysis (described in materials and methods). The error bars represent the mean deviation for two replicates

removed 1200 mg L^{-1} and 200 mg L^{-1} of APAP in MSM, respectively (Chopra and Kumar 2020; Palma et al. 2021). Murdoch and Hay (2015) studied the ability of *Variovorax* sp. Ibu-1 to use 200 mg L^{-1} of **ibuprofen** as a carbon source. It is important to highlight that previously studied genera, except *Bacillus*, have not been identified as having APAP degradation capacities.

4-hydroquinone biodegradation in solution by *M. aubagnense* HPB1.1

The Poderosa mine isolate *M. aubagnense* HPB1.1 was the only one selected as a potential H2Q-removing bacterium for the biodegradation tests in liquid MSM monitored by HPLC analysis in the current study. As shown in Fig. 3S, H2Q removal in the culture with HPB1.1 strain was not associated with a brownish/pinkish coloration, while in the negative control this coloration, which appeared very quickly after H2Q solubilization and before incubation in the dark, stayed during all the incubation time. This coloration indicates H2Q oxidation in contact with air and light (Enguita and Leitão, 2013) and/or that the medium's physical–chemical characteristics potentiated H2Q oxidative polymerization (Zhang et al. 2012a). Therefore, it was important to try to understand and monitor as much as possible the abiotic H2Q transformation.

It is known that H2Q autoxidation can occur in water at room temperature, resulting in the production of 1,4-benzoquinone (BQ); even if at a small rate without the addition of a catalyst (Owsik and Kolarz 2002; Chunmei et al. 2015). In detail, it has been suggested that the autoxidation of H2Q to quinones (Q) occurs in free-radical chain (cyclic) processes (Roginsky and Barsukova 2000). On the other hand, it is known that the simplest quinone, the 1,4-benzoquinone (BQ), is light sensitive, and studies on its photolysis have shown that upon excitation by visible light it can go through two pathways of reactions that both result in 2-hydroxy-1,4-benzoquinone (*2*-OH-BQ) and H2Q (Ononye et al. 1986; Von Sonntag et al. 2004). Moreover, it has also been shown that the 2-hydroxy-1,4-benzoquinone (2- OH -BQ) can be further oxidized to 2,5-dihydroxy-1,4-benzoquinone (2,5-OH-BQ) (Kurien and Robins 1970; Fónagy et al. 2021). Figure 4S illustrates the reactions occurring in the two possible pathways. Thus, when H2Q is solubilized in water and exposed to light, four main compounds can be present at a certain equilibrium: hydroquinone (H2Q), 1,4-benzoquinone (BQ), 2-hydroxy-1,4-benzoquinone (2- OH-BQ) and 2,5-dihydroxy-1,4-benzoquinone (2,5-OH-BQ). Interestingly, these four compounds have different absorption spectra (Fónagy et al. 2021), which can be used for identification. H2Q has a characteristic absorption peak at ~ 288 nm, BQ has a major absorption peak at ~ 246 nm, 2-OH-BQ has a major characteristic peak at ~ 256 nm and another wider but lower peak at ~ 485 nm, 2,5-OH-BQ has a major characteristic peak at ~ 300 nm. For example, Rouco et al. (2018) evaluated the reduction of p-benzoquinone (peak at 246 nm) into H2Q (peak at 290 nm) by spectrophotometric monitoring of the reaction using the spectra.

In our study, when H2Q was dissolved in liquid MSM medium, abiotic transformation started to occur as indicated by the brownish/pinkish coloration developed and by the observation of more than one peak by HPLC analysis (details in methods). Thus, a reference material (Hydroquinone TraceCERT®, 74,347—Supelco) was used to confirm that the main peak (retention times (RT):—RT ~ 4.1 min) was in fact H2Q. The initial H2Q concentration was ~ 9.4 mg L^{-1} and it dropped to non-detected values in four days in the test with *M. aubagnense* HPB1.1, while just a small decay from ~ 9,4 to 7.9 mg L^{-1} occurred in the negative (abiotic) control (Fig. 3), indicating that most of the H2Q removal in the culture was due to biotic factors.

In what concerns H2Q transformation products, three peaks (apart from the H2Q peak) were detected: one with a RT of ~ **4.9** min, with the typical absorption spectra of 2,5-OH-BQ; the second with a RT of ~ **5.4** min, also with the typical absorption spectra of 2,5-OH-BQ; and the third with a RT ~ **6.8** min, with the typical absorption spectra of BQ (Fig. 4). According to the absorption spectra, one of the two peaks at ~ 4.9 min and ~ 5.4 min should correspond to 2,5-OH-BQ, while the other is probably another hydroxybenzoquinone isomer also with two hydroxyls. During the H2Q biodegradation test the peak at RT ~ 5.4 min was not detected. Yet, interestingly, the peaks at RT ~ 4.9 min and RT ~ 6.8 min (2,5-OH-BQ and BQ, respectively) increased in the negative (abiotic) control and decreased to non-detection in the pure culture of M. aubagnense HPB1.1 (Fig. 5). This is another evidence that the strain degraded H2Q (and/

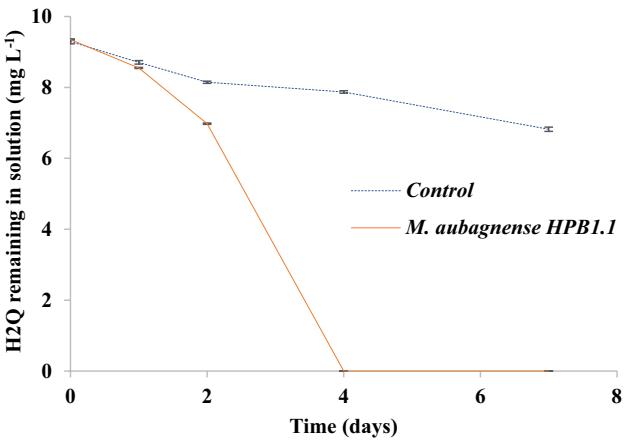
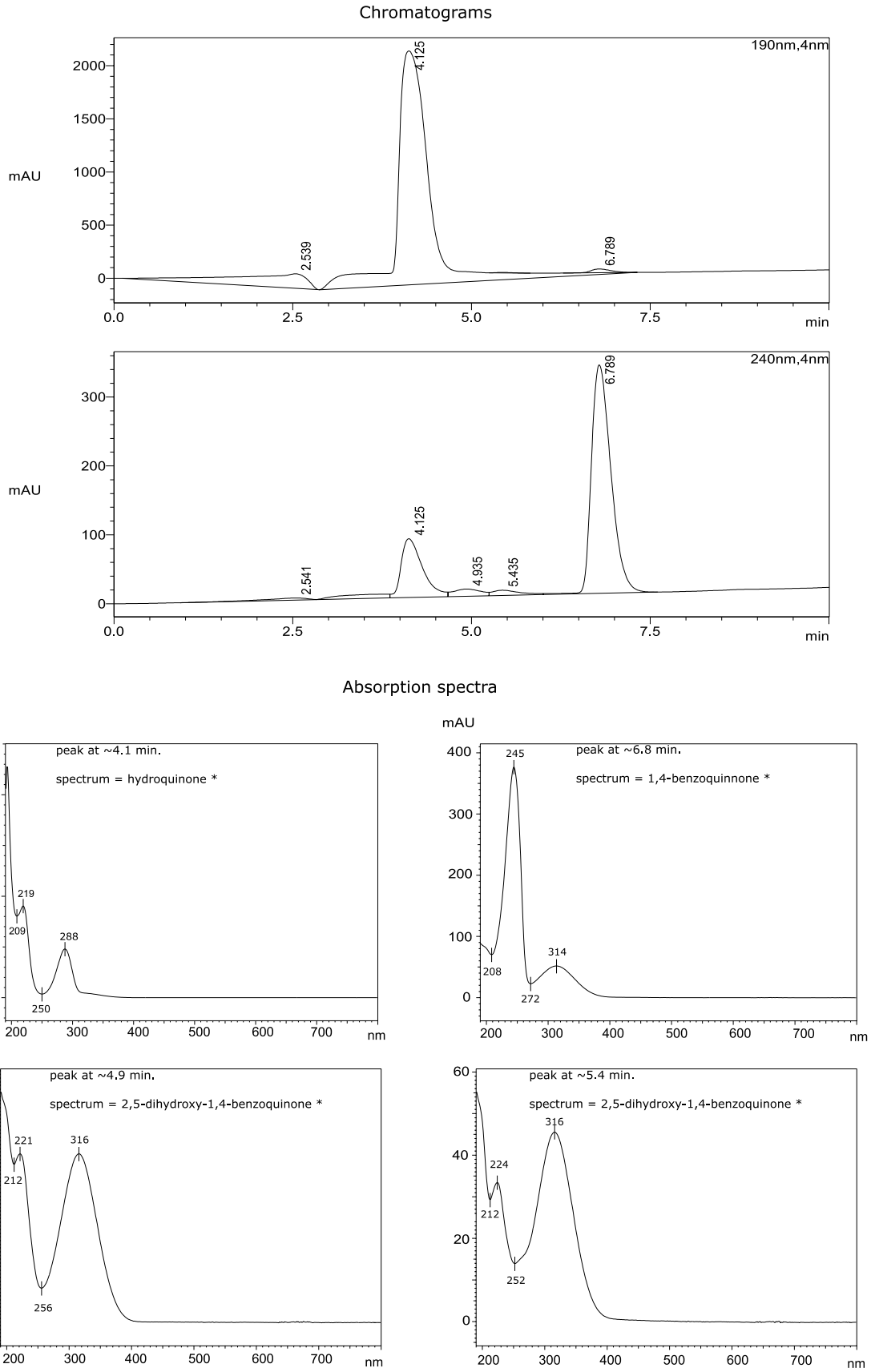


Fig. 3 Hydroquinone (H2Q) concentration in MSM-H2Q (10 mg L^{-1}) (Control) and after inoculation of M. augbanense HPB1.1. The error bars represent the mean deviation for two replicates


Chromatograms
190nm,4nm
mAU
2000
1500
1000
500
0
2.539
4.125
6.789
0.0
2.5
5.0
7.5
min
240nm,4nm
mAU
300
200
100
0
2.541
4.125
4.935
5.435
6.789
0.0
2.5
5.0
7.5
min
Absorption spectra
mAU
peak at ~4.1 min.
spectrum = hydroquinone *
2500
2000
1500
1000
500
0
209
219
250
288
200
300
400
500
600
700
nm
mAU
peak at ~6.8 min.
spectrum = 1,4-benzoquinnone *
400
300
200
100
0
208
245
272
314
200
300
400
500
600
700
nm
peak at ~4.9 min.
spectrum = 2,5-dihydroxy-1,4-benzoquinone *
60
40
20
0
212
221
256
316
200
300
400
500
600
700
nm
peak at ~5.4 min.
spectrum = 2,5-dihydroxy-1,4-benzoquinone *
60
40
20
0
212
224
252
316
200
300
400
500
600
700
nm

◂**Fig. 4** Hydroquinone solubilized in the aqueous Mineral Salt Medium (MSM) analyzed by HPLC (details in methods). *Hydroquinone transformation products with matching absorbance spectra (Rouco et al. 2018; Fónagy et al. 2021)

or its transformation products) and is in accordance with the absence of coloration in the culture after incubation and with the appearance and maintenance of coloration in the negative control.

The ability of *M. aubagnense* HPB1.1 strain to degrade H2Q was indeed also corroborated by an increase in the optical density (600 nm) due to bacterial growth in test in liquid MSM with H2Q as the sole carbon source (Fig. 5S). The growth was not very sharp, due to the fact that the test started with an already relatively high OD600 (~0.8) and also because the initial amount of carbon and energy source was low (the concentration of hydroquinone was only 9.4 mg L^{-1}), but there was clearly growth. Additionally, an adsorption/absorption study was conducted to rule out possible adherence/accumulation in the bacterial cells, and the absence of H2Q or any transformation product in the cells finally confirmed that H2Q was indeed degraded as a result of the presence of *M. aubagnense* HPB1.1 strain.

It is important to highlight the problems generated by the presence of H2Q in the environment since it is a ubiquitous pollutant and is considered the most reactive and toxic intermediate of the quinone species (North et al. 2011). Despite the ubiquity and toxicity of H2Q, few bacteria have been reported as H2Q-degrading. Bae et al. (1996) described *Arthrobacter ureafaciens* CPR706 as para-substituted phenols degrading via the H2Q pathway. Later, *Moraxella* sp. (Spain and Gibson 1991), *Pseudomonas* sp. WBC-3 (Chen et al. 2009), and *Pseudomonas* sp. 1–7 (Zhang et al. 2012b) were able to degrade H2Q formed in para-nitrophenol degradation. According to Vargas-Ordoñez et al. (2023), *Stutzerimonas stutzeri* CSW02 and *Pseudomonas extremaustralis* CSW01 showed an ability to metabolize H2Q from APAP biodegradation. However, none of these works were conducted using H2Q as the initial compound. For the reasons already mentioned above, this study is especially significant in the biodegradation field. In addition, it is the first time that *Mycolicibacterium* sp. is reported as a degrader of H2Q. Not only that, but it is also the first time it is been reported as a pharmaceutical degrader.

In silico prediction of genes involved in the paracetamol degradation pathway

Our novel identification of the *M. aubagnense* HPB1.1 strain as a pharmaceutical degrader, namely by degrading H2Q which is an intermediate metabolite of APAP and other phenolic compounds, led us to sequence its genome. The assembled genome of *M. aubagnense* HPB1.1 was deposited in NCBI Genbank (accession numbers: CP122994—CP12299). The genome assembly consists of 6 contigs: one is the circular chromosome (CP122994) with a size of 5.734.470 bp, two are linear sequences probably from plasmids whose circular sequence could not be closed (CP122996, CP122997) of 181.565 bp and 99.865 bp, and three are circular plasmid sequences (CP122995, CP122998, CP122999) of 168.657 bp, 23.691 bp, and 16.144 bp respectively. The HPB1.1 genome presents a total size of 6.224.392 bp and has 66,4% Cytosine (G-C) content and 5838 identified genes.

50 genes were identified in the genome of the HPB1.1 strain as responsible for aromatic compound metabolism (Fig. 6S, boxed in red). Specifically, these genes are related to salicylate ester, quinate, and biphenyl degradation, catechol and protocatechuate branch of the beta-ketoadipate pathway, salicylate and gentisate catabolism, homogentisate pathway and degradation of gentisate. Some of the pathways above-mentioned have been related to APAP degradation pathway. Indeed, it is for long known that the biodegradation of APAP in microbes proceeds via 4-aminophenol to hydroquinone and then conversion to catechol with subsequent ring fission (e.g., Wu et al. 2012; Chacón et al. 2022; Zhang et al. 2013). Furthermore, studies have shown that there is a high diversity of bacterial species with enzymes responsible for metabolizing the intermediate products of 2,5-dihydroxy-6-oxo-2,4-hexadienoic generated through the beta-ketoadipate pathway in the tricarboxylic acid (TCA) cycle that is involved in the microbial degradation of paracetamol (Rios-Miguel et al. 2022). In general, amidases, deaminases, hydroxylases, and dioxygenases have been proposed in a review article as enzymes potentially involved in the biodegradation pathway of APAP (Grignet et al. 2022).

The current study embraces and discusses the challenges of researching the genome of a bacterial strain showing the capacity of APAP degradation, aiming to identify candidate genes encoding potential enzymes that could be involved in the biodegradation pathway of this drug. To achieve this, an in silico analysis was conducted to predict the genes associated with APAP biodegradation in the genome of the HPB1.1 strain, employing sequence homology comparison with enzymes reported for the degradation of main intermediate aromatic compounds occurring during APAP biodegradation. The details on the selection of each reference protein are described below, after this next paragraph with a brief overview of the study carried out on the obtained protein alignments.

Usually, the percentage of identity required to confirm that two proteins in different bacteria have the same function can vary depending on several factors, including the protein level of conservation and the specific functional regions involved. Generally, a higher percentage of identity suggests a higher likelihood of functional similarity between the proteins. Nevertheless, there is no set threshold to establish the

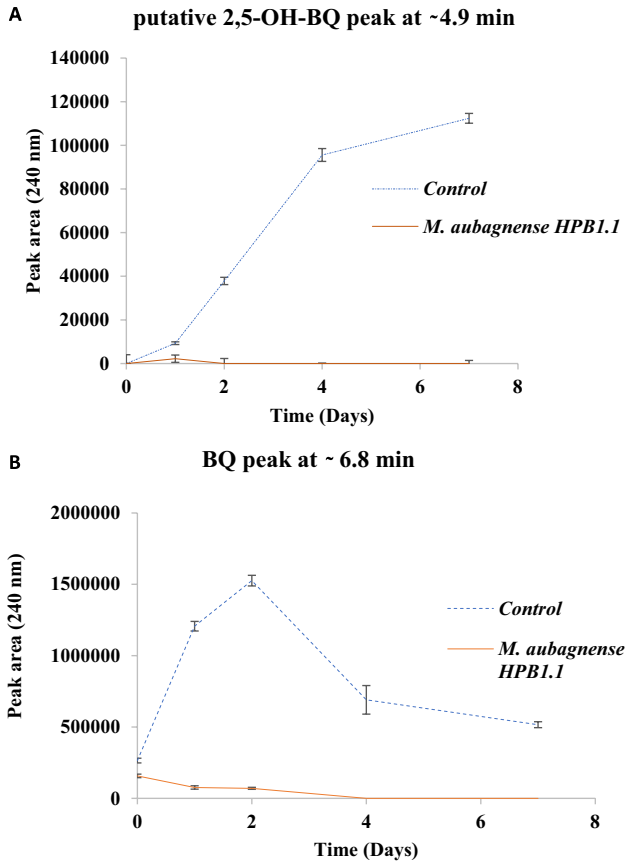


Fig. 5 The peak area of A) putative 2,5-dihydroxy-1,4-benzoquinone (2,5-OH-BQ) and B) 1,4-Benzoquinone (BQ) in solution throughout the biodegradation process of Hydroquinone (H2Q) in presence of M. aubagnense HPB1.1. The error bars represent the mean deviation for two replicates

accurate percentage of identity needed to determine functional equivalence. According to Rost (1999), sequence alignments are considered capable of unambiguously distinguishing between pairs of proteins of similar and non-similar structure when the sequence identity by pairs is high (> 40% for long alignments). However, the percentage of identity between proteins with similar function can in some cases be lower than 40% due to high evolutionary divergence in sequence regions non-essential for structural and functional maintenance. Todd et al. (2001) studied the variation in enzyme function in different groups of superfamilies, concluding that in the case of single and multi-domain proteins, there is minimal variation in the Enzyme Commission (EC) number (a number associated with a specific enzyme-catalyzed reaction) when the sequence identity exceeds 40%, and therefore when that is the case highly probably it maintains the same function. On the contrary, for proteins that share less than 30% sequence identity, significant functional variation occurs, and below this threshold, structural data becomes essential to understand the functional similarities or differences. On the other hand, as reported by Galperin and Koonin (2012), enzymes belonging to a superfamily typically exhibit shared sequence motifs, and essential active site residues, and often have predicted reaction mechanisms. For these reasons, an analysis of the catalytic domains has been carried out in the identified proteins whose alignment's identity percentages were lower than 40%.

Amidases are considered the most likely candidates responsible for the first step of the APAP biodegradation pathway (Lee et al. 2015; Rios-Miguel et al. 2022). These enzymes are known to catalyze the hydrolysis of amide bonds ($CO–NH_2$) conducting the formation of ammonia and carboxylic acid (Wu et al. 2020). In the case of APAP degradation by microbial organisms, amidases have been described as responsible for transforming APAP into 4-AP. Therefore, characterized aryl- amidases from *Pseudomonas* sp., *Paracoccus huijuniae* and *Ochrobactrum* sp. (Table 2–three first lines), all of them with ability to use APAP as substrate (Ko et al. 2010; Zhang et al. 2012c; Yun et al. 2017), were used as reference proteins to search for proteins in the genome of the HPB1.1 strain potentially with the same function. This bioinformatic analysis revealed identity percentages ranging from 28 to 38% to the same protein, annotated as Aspartyl-tRNA(Asn) amidotransferase subunit A in the HPB1.1 genome. In addition, the specific residues involved in the catalytic step of the pathway studied by Lee et al. (2015) were also examined in our alignment (Fig. 7S). The alignment exhibits a conserved catalytic triad (Ser^{163}–Ser^{187}–Lys^{84}, in red bold) and Gly/Ser-rich motif (GGSSGG, yellow highlight). This evidence strongly supports this enzyme in the genome of the HPB1.1 strain is functional.

The search for a candidate amidohydrolase was carried out using as query this enzyme annotated in the genome of *Mycolicibacterium mucogenicum* DSM 44124 (GenBank: ANBS01000023.1) (Behra et al. 2019) as a protein. This species is associated with a wide spectrum of clinical diseases and is commonly implicated in outbreaks of infection resulting from contaminated hospital equipment and water sources (Adékambi 2009), which is an indication of resilience and resistance/adaptation to recalcitrant compounds. Nowadays, it is becoming recognized that *Mycobacterium* species exhibit high bioremediation potential for the degradation of a wide range of organic pollutants, including polycyclic aromatic hydrocarbons (PAHs), by converting them into simpler, non-toxic compounds (Deng et al. 2023; Kim et al. 2010). Indeed, the isolation of *M. aubagnense* HPB1.1 as APAP and H2Q degrader strain reported in this work is another contribution to this evidence. The search against the *M. aubagnense* HPB1.1 genome revealed the presence of a putative deaminase gene, with an alignment exhibiting 92% identity to a protein annotated as exoenzymes regulatory protein AepA precursor. AepA was first described

Table 2 Information about the alignment of amidase, deaminases, hydroxylase, and dioxygenase reference proteins, known to degrade paracetamol to proteins annotated in the genome of *M. aubagnense* HPB1.1

	REFERENCE PROTEIN DATA			ALIGNMENT DATA			ANNOTATED PROTEIN DATA		
	Protein (Accession number)	Function	Bacterium	Coverage (%)	Identity (%)	E-value	Contig	Peg annotation	Putative function
Amidases	ACP39716.2	aryl acylamidase	*Pseudomonas* sp.	96	31	$2e^{-36}$	ctg.s1.000000F	3681	* Aspartyl-tRNA(Asn) amidotransferase subunit A
	AFC37599.1	aryl-amidase A	*Paracoccus huijuniae*	52	38	$3e^{-24}$			
	ANS81375.1	aryl-amidase	*Ochrobactrum* sp. PP-2	97	28	$9e^{-25}$			
Deaminases	KAB7755695.1	amidohydrolase	*Mycolicibacterium mucogenicum* DSM 44124	100	92	0	ctg.s1.000000F	1068	*Exoenzymes regulatory protein AepA precursor
Hydroxilases	AKA64675.2	aromatic-ring-hydroxylating oxygenase alpha subunit	uncultured bacterium	90	30	5e-40	ctg.s1.000000F	700	*Putative dioxygenase hydroxylase component
	AYA78733.1	aromatic-ring-hydroxylating oxygenase beta subunit	uncultured bacterium	60	28	4e-07	ctg.s1.000000F	701	*Benzoate 1,2-dioxygenase beta subunit
Dioxygenases	KAB7760265.1	extradiol ring-cleavage dioxygenase III subunit A	*Mycolicibacterium mucogenicum* DSM 44124	95	92	0	ctg.s1.000000F	1823	*fumarate reductase/succinate dehydrogenase flavoprotein, N-terminal:FAD dependent oxidoreductase
	KAB7760264.1	extradiol ring-cleavage dioxygenase III subunit B	*Mycolicibacterium mucogenicum* DSM 44124	95	82	0	ctg.s1.000000F	1822	*hypothetical protein
	ANH99603.1	intradiol ring-cleavage dioxygenase	*Pseudomonas koreensis*	99,7	31	$2e^{-09}$	ctg.s2.000001F	5638	Protocatechuate 3,4-dioxygenase beta chain

*Candidate proteins most likely to be responsible for APAP biodegradation based on identity and coverage percentage.

Peg (Protein Encoding Gene) from RAST server.

ANNOTATED PROTEIN DATA correspond to the annotation performed with RAST server.

as regulating the synthesis of degrading enzymes, but then has been proposed as being an amidohydrolase (Kõiv et al. 2010). These findings suggest the presence of the amidohydrolase enzyme in the genome of the HPB1.1 strain, enabling the bacteria to convert *4*-AP into H2Q (Rios-Miguel et al. 2022).

Subsequently, H2Q has the potential to undergo hydroxylation, resulting in the formation of hydroxyquinol, with the addition of hydroxyl groups to the aromatic ring. The H2Q hydroxylation can be conducted by ring-hydroxylating dioxygenases or monooxygenases (RHOs). RHOs are composed of two protein components: an electron transport chain and an oxygenase. In this study, the possible presence of genes with hydroxylase activity has been studied using as reference the aromatic-ring-hydroxylating oxygenase alpha and beta subunits from an uncultured bacterium, which were cloned by Musumeci et al. (2019) in *Escherichia coli* for functional studies and showed activity for different substrates with aromatic rings. The results showed 30% and 28% identity to the consecutively annotated dioxygenase hydroxylase component and benzoate 1,2-dioxygenase beta subunit in the HPB1.1 strain genome, respectively. The catalytic domain of the oxygenase alpha subunit and related aromatic ring hydroxylating dioxygenases were checked using the Conserved Domain Database (CDD) to confirm the ring-hydroxylating oxygenase activity. The results showed the presence of active sites involved in the investigated function (Fig. 8S).

Dioxygenases were the last protein group studied. Several dioxygenases have been described as responsible for catalyzing the opening of the aromatic ring in the catabolism of aromatic compounds (Semana and Powlowski 2019; Mishra et al. 2020; Xue et al. 2021). However, little is known about their involvement in APAP biodegradation (Żur et al. 2018; Rios-Miguel et al. 2022). The presence of intradiol and extradiol ring-cleavage dioxygenase enzymes in the HPB1.1 genome was analyzed first using a reference protein from *Pseudomonas* species since this genus is long known to have APAP degrading capacity (e.g., Vargas-Ordoñez et al. 2023). The alignment of intradiol ring-cleavage dioxygenase from *Pseudomonas koreensis* revealed a protein in the HPB1.1 genome with aligned with very high coverage (99.7%) and 31% identity. Moreover, the extradiol ring-cleavage dioxygenase III subunits A and B described for *M. mucogenicum* DSM 44124 were also used for searches and the results

revealed two consecutive proteins exhibiting high percentages of identity (92% and 82%, respectively) (Table 2), which indicates the presence of functional extradiol ring-cleavage dioxygenase in the studied bacterium. These proteins correspond to the proteins annotated as fumarate reductase/succinate dehydrogenase flavoprotein and hypothetical protein in the annotation of HPB1.1 strain. Extradiol ring-cleavage dioxygenase III catalyzes the incorporation of both atoms of oxygen from O_2 into catechol derivatives, resulting in ring-open, muconic semialdehyde adducts that are readily degraded to TCA (Lipscomb 2008).

This genomic study presenting candidate genes for putative enzymes that catalyze the main steps of the APAP degradation pathway, together with the observed ability of *M. aubagnense* HPB1.1 to grow either with APAP or H2Q as the sole carbon source, is the starting point for future works on gene overexpression or gene knockouts (also known as gene deletion or gene inactivation) to evaluate the real role of the identified candidate genes in the biodegradation of APAP and/or its intermediate metabolite H2Q. It is also shown that the distribution of the candidate proteins that are proposed as being responsible for APAP biodegradation are all located in the ctg.s1.000000F (Table 2) corresponding to the bacterial chromosome. If the catabolic gene conferring the APAP degrading capacity were all located on a plasmid, the strain could have the potential application in studies on conjugative plasmid transfer to native bacteria on bioreactors to enhance biodegradation. Nevertheless, the location in the chromosome makes this ability more robust in the strain than if it was partially or fully located on a plasmid, which the bacteria could lose in nonselective environments.

Conclusions

Several APAP-degrading bacterial strains were proposed in this work. We focused our studies on the *M. aubagnense* HPB1.1 strain, isolated from a Poderosa mine sample by selective enrichment in the presence of H2Q, which was able to use APAP and H2Q as its sole energy and carbon source, despite being from a species not previously reported as having a pharmaceutic-degrading capacity. Two metabolites (*2,5*-OH-BQ or another hydroxybenzoquinone, and BQ) were detected throughout the H2Q biodegradation in solution, and their degradation was confirmed when the HPB1.1 strain was present. To complement the evidence showing such biodegradation capacity, a genome-based analysis of *M. aubagnense* HPB1.1 was performed to suggest candidate genes putatively involved in APAP degradation, as the starting point for future works on gene function studies and genetic bioaugmentation aiming wastewater treatment improvement. We identified one aryl acylamidase enzyme as possibly being responsible for catalyzing the first step of the APAP biodegradation pathway. Thereafter an amidohydrolase likely responsible to transform 4-aminophenol into H2Q. Later in the pathway, two subunits of a hydroxylase are suggested as potentially converting H2Q into hydroxyquinol. Finally, dioxygenases are pointed as probably responsible for the aromatic ring opening, a step in the pathway which is crucial to the complete degradation of the initial compound into less toxic non-aromatic compounds.

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Author contributions Alba Lara-Moreno: methodology, investigation, formal analysis, data curation, writing—original draft. Fatma El-Sayed: experimental work, investigation. Cymon J. Cox: bioinformatics for genome assembly and manuscript revision. Maria C. Costa: supervision, funding acquisition. Jorge D. Carlier: methodology, investigation, supervision, funding acquisition, review & editing.

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Declarations

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this manuscript.

Informed consent We affirm that all the authors have seen, prepared, and agreed to the submission of the paper and their inclusion of name(s) as co-author(s). We also declare that there are no conflicts of interest for the same.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

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