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RESEARCH ARTICLE

Isolation of a diphenylamine-degrading bacterium and characterization of its metabolic capacities, bioremediation and bioaugmentation potential

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Abstract The antioxidant diphenylamine (DPA) is used in fruit-packaging plants for the control of the physiological disorder apple scald. Its use results in the production of DPA-contaminated wastewater which should be treated before finally discharged. Biological treatment systems using tailored-made microbial inocula with specific catabolic activities comprise an appealing and sustainable solution. This study aimed to isolate DPA-degrading bacteria, identify the metabolic pathway of DPA and evaluate their potential for future implementation in bioremediation and biodepuration applications. A *Pseudomonas putida* strain named DPA1 able to rapidly degrade and utilize DPA as the sole C and N source was enriched from a DPA-contaminated soil. The isolated strain degraded spillage-level concentrations of DPA in liquid culture (2000 mg L^{-1}) and in contaminated soil (1000 mg kg^{-1}) and metabolized DPA via the transient formation of aniline and catechol. Further evidence for the bioremediation and biodepuration potential of the *P. putida* strain DPA1 was provided by its capacity to degrade the post-harvest fungicide *ortho*-phenylphenol (OPP), concurrently used by the fruit-packaging plants, although at slower rates and DPA in a wide range of pH (4.5–9) and temperatures (15–37 °C). These findings revealed the high potential of the *P. putida* strain DPA1 for use in future soil bioremediation strategies and/or as start-up inocula in wastewater biodepuration systems.

Keywords Diphenylamine · Fruit-packaging industry · *Pseudomonas putida* · Pesticide biodegradation · Bioremediation · Biodepuration

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Introduction

Diphenylamine (*N-phenylbenzenamine*, DPA) is an antioxidant compound used in fruit-packaging industry to control the physiological disorder scald on apples and pears, which diminishes the market value of pome fruits during storage (Jung and Watkins 2008; EC 2012). DPA is authorized for use in several countries including USA, Canada, Australia, Chile, Argentina, Uruguay, South Africa and in all countries which have adopted the Codex standards. In contrast, in 2009, the European Commission (EC) did not grant authorization to DPA. This decision was based on the identification of unacceptable risks for consumers stemming from the lack of data on the presence and toxicity of unidentified metabolites and the possible formation of nitrosamines during storage of the active ingredient or during apples and pears processing (EC 2012). However, the lack of equally effective alternatives to DPA led several member states like France, Ireland, UK, Portugal, Spain, Italy and Greece to grand exemption authorization to DPA for a 120-day period. This was imposed for the first time in 2012, and since then, DPA is still used in some of the above EU member states under the same exemption authorization scheme.

DPA is generally applied on apples and pears by drenching at concentrations ranging from 400 to 2000 mg L^{-1} (EC 2012). This mode of application results in the formation of large wastewater volumes with high DPA concentration. In order to address this issue, the European Food Safety Agency (EFSA 2012) imposed that "management measures tailored to local practice and legislation need to be put in place to control the waste disposal of spent application solution (formed during DPA treatments) and prevent accidental spillage entering sewers or surface water drains". Considering the high toxicity of DPA on aquatic organisms including fish (*Oncorhynchus mykiss* EC_{50} 2.2 mg L^{-1}), algae (*Selenastrum capricornutum* biomass and growth rate EC_{50} 0.18 and 0.30 mg L^{-1}, respectively) and invertebrates (*Daphnia magna* EC_{50} 1.2 mg L^{-1}) (Drzyzga 2003; EC 2008), the direct environmental release of DPA-containing wastewater without any prior treatment entails a serious risk for the environment and should be mitigated.

Several attempts have been made for the development of treatment systems for the detoxification of DPA-contaminated wastewater. These included the use of a filtering system based on a mixture of peat moss, manure, clay and dolomite sand (Flaim and Toller 1989) or the use of fungal laccases (Saha et al. 2008). Although efficient, these methods were never adopted by the fruit-packaging industry due to their high cost or high labour requirements for handling high wastewater volumes (Karanasios et al. 2012). This has forced most fruit-packaging industries to discharge their DPA-contaminated wastewater into nearby abandoned fields and evaporation ponds or directly into the municipal sewage treatment system, increasing the likelihood for environment contamination and degradation of ecosystems quality. Although DPA is not persistent in the soil environment (Karas et al. 2015), the continuous disposal of DPA-contaminated effluents in soil could lead to the accumulation of DPA which need to be removed or else the contaminated soil could further acts as a sink for the subsequent transfer of DPA residues in adjacent natural water bodies (Drzyzga 2003). Alternatively, those wastewaters are collected by certified companies which treat them ex situ as toxic wastes, a particularly costly procedure for the owners of fruit-packaging plants (Karanasios et al. 2012). Thus, there is an urgent need for the development and implementation of sustainable and cost-effective methods for the treatment of DPA-contaminated wastewater. Biological treatment systems based on specialized DPA-degrading inocula could be a solution for the treatment of those wastewaters.

According to the Technical Guidance Document on Risk Assessment, DPA is regarded as not readily biodegradable (EC 2008). This contradicts with the appreciable DPA microbial degradation observed in a sewage sludge experiment (Gardner et al. 1982), in anoxic sediment–water batch enrichments and in cultures of sulphate-reducing bacteria (Drzyzga et al. 1996; Drzyzga and Blotevogel 1997). Christodoulatos et al. (1997) first reported the degradation of DPA (15 mg L^{-1}) by three *Pseudomonas* strains, obtained from the American Type Culture Collection (ATCC), in minimal media where the pesticide constituted the sole C source; however, no other information regarding the degrading characteristics of the tested bacteria were provided. Subsequent studies by the same authors in a pilot bioreactor inoculated with activated sludge showed a rapid degradation of DPA (25.4–57.1 mg L^{-1}) with an average $t_{1/2}$ of 1.40 days. In a more recent and the only comprehensive study regarding microbial degradation of DPA, Shin and Spain (2009) reported the isolation of two DPA-degrading strains, a *Burkholderia* sp. and a *Ralstonia* sp., and provided evidence for the metabolic pathway of DPA. It was shown that DPA was metabolized to catechol and aniline, which were further transformed via relevant pathways that are ubiquitous among soil bacteria. Apart from bacterial degradation, white-rot fungi like *Trametes versicolor* and *Pleurotus ostreatus* were able to rapidly degrade DPA via their lignolytic enzymatic system (Karas et al. 2011). However, the limited capacity of white-rot fungi to survive and grow in wastewater treatment systems prohibits their use as microbial inocula for the treatment of those effluents (Gao et al. 2008).

Based on the limited information available in the literature regarding the microbial degradation of DPA and the urgent need for DPA-degrading microorganisms which could act as starting inocula in wastewater treatment systems and for soil bioremediation, we aimed to isolate and identify bacteria able to rapidly and effectively degrade DPA. Subsequent focus was given to the characterization of the degrading capacities of the isolated microorganisms to get an overview of their application potential in wastewater biodepuration and soil bioremediation schemes.

Materials and methods

Pesticides and other substrates

Diphenylamine (Pestanal®, 99.9 % purity) was purchased from Sigma-Aldrich. A stock solution of 1000 mg L^{-1} in methanol was prepared and serial dilutions were used for analytical purposes. A stock solution of DPA in water (100 mg L^{-1}) was also prepared; it was filter sterilized (0.22 μm, MS® PES Syringe Filter) and used for the preparation of DPA-amended media. Due to the limited water solubility of DPA, a commercial formulation (No Scald DPA 31.8 EC, DeccoIberica, Spain, composition: 31.8 %*w*/*v* DPA–68.8 %*w*/*w* additives like emulsifying agents) was used for the preparation of aqueous DPA solutions at concentrations above 100 mg L^{-1} which were subsequently filter sterilized. Alternatively, DPA solutions in DMSO (20 g L^{-1}) were prepared and filter sterilized. In cases where DMSO solutions were used, its amount in the medium was adjusted to 0.3 %. Preliminary studies in our laboratory showed that no

inhibitory effects on the degradation and survival of the DPA-degrading isolate were evident at levels of DMSO in the medium reaching to 7.5 %.

Other molecules used in the present work were catechol (≥99 % purity, Sigma-Aldrich), aniline (≥99 % purity, ChemLab Supply) and *ortho*-phenylphenol (OPP) (Pestanal®, 99.9 % purity, Sigma-Aldrich). For those compounds, stock solutions in methanol (1000 mg L^{-1}) were used for analytical purposes, whereas water (100 mg L^{-1} for OPP) and DMSO solutions (10 g L^{-1} for aniline and catechol) were used for experiments in liquid cultures.

Pesticide and metabolite residue analysis

Chemicals were extracted from liquid media by mixing 0.5 mL of culture with 1 mL of methanol (HPLC Grade, Merck) in glass vials and vortexing for 30 s at maximum speed. After 3 min of centrifugation at maximum speed, the clear supernatant was collected and stored at −20 °C until downstream analysis. Recovery tests with media containing each chemical at three concentration levels (0.2, 2 and 20 mg L^{-1}) showed recoveries higher than 80 %.

DPA was extracted from soil by mixing 10 g of soil with 25 mL of acetonitrile (ACN). The mixture was agitated for 1.5 h in an orbital shaker at 200 rpm and then centrifuged at 11,000 rpm for 5 min. The supernatant was collected in glass bottles and filtered (45 μm, PTFE Syringe Filter) before analysis in an HPLC system. Recovery tests at three concentration levels (0.2, 2 and 20 mg kg^{-1}) showed recoveries higher than 90 % in all cases.

Residues of the chemicals studied were analysed in an HPLC-UV system equipped with a CNW Athena RP C18 150 mm×4.6 mm column. DPA residues were determined at 210 nm using a mobile phase of 70:20:10 ACN:water:methanol (by volume). Under these conditions, DPA showed a retention time (Rt) of 3.5 min. Catechol was eluted using a mobile phase of 60:40 water:ACN (by volume)+0.1 % acetic acid and its residues were determined at 276 nm (Rt=2.5 min). Aniline was eluted using a mobile phase of 60:40 ammonium acetate solution (0.4 g L^{-1} at pH 4.2):ACN (by volume) and detection was achieved at 254 nm (Rt=3.4 min). Finally, OPP residues were determined at 254 nm with a liquid phase composed of 70:29.5:0.5 ACN:water:25 % NH_3 solution (by volume) (Rt=3.4 min). In all cases, the flow rate of the mobile phase was 1 mL min^{-1}.

Growth media

Two selective media were used for the isolation of DPA-degrading bacteria: a mineral salt medium supplemented (MSMN, N added as NH_4Cl) or not supplemented (MSM) with nitrogen. In these media, the pesticide constituted the sole C or the sole C and N source, respectively. Both media were prepared as described previously by Karpouzas and Walker (2000). The two media were amended with DPA using a 100 mg L^{-1} filter-sterilized aqueous stock solution aiming to a final concentration of 20 mg L^{-1} unless otherwise stated. Agar plates of the corresponding media+DPA (20 mg L^{-1}) were prepared by the addition of 15 g L^{-1} of agar. Other media used were the Luria-Bertani (LB) medium (10 g L^{-1} NaCl, 10 g L^{-1} bacteriological peptone, 5 g L^{-1} yeast extract) and the biobed extract medium (BEM). For BEM preparation, 100 g of organic substrate (50 % spent mushrooms substrate, 25 % soil and 25 % straw, by volume) was mixed with 1 L of deionized water, and after a first heat-sterilization and centrifugation, the supernatant was re-autoclaved and further used (Perruchon et al. 2015).

Enrichment and isolation of DPA-degrading bacteria

A soil from a wastewater disposal site located nearby a fruit-packaging plant in the area of Agià, Larissa, Greece, was used as a source of DPA-degrading bacteria. According to the owner of the fruit-packaging plant, the specific site has been used for the disposal of wastewaters for several years, although the levels of DPA concentrations in the soil were low (unpublished data). Prior to the onset of the enrichment cultures, the soil was treated with a fresh addition of DPA (10 μg g^{-1}) and incubated under aerobic condition in the dark at 25 °C for 7 days to stimulate the specific DPA-degrading soil microbiota. The enrichment culture method in MSM and MSMN, as described by Karpouzas et al. (2000), was used for the isolation of DPA-degrading bacteria. At the point of 50 % degradation of DPA in the fourth enrichment cycle, a 10-fold dilution series was spread on DPA-containing (20 mg L^{-1}) MSM or MSMN agar plates. Upon incubation at 26 °C for 4–5 days, growing colonies were selected, inoculated in fresh MSMN+DPA and MSM+DPA liquid media and were analysed for DPA degradation. Cultures exhibiting >50 % DPA reduction in 7 days were considered as positive and were plated on LB and MSM or MSMN+DPA agar plates to verify purity. The bacteria that appeared as pure in plates were processed for DNA extraction.

Identification and phylogenetic analysis of the DPA-degrading bacteria

DNA extraction from bacteria was performed with the Purelink Genomic DNA Minikit (Invitrogen Life Technologies). The primer pair 8f-1512r which amplifies almost the full size of the 16S ribosomal RNA (rRNA) gene (1500 bp, Felske et al. 1997) was used for the phylogenetic analysis of the isolated bacteria. Amplification was carried out in 25-μL reactions containing 1 U of DyNAzyme™ EXT (Finnzymes), 0.2 μM of each primer, 1× buffer (DyNAzyme™ EXT buffer), 1.5 mM of $MgCl_2$ and

200 μM of each dNTPs. The PCR thermal cycling conditions were 95 °C for 5 min, followed by 25 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, with a final extension of 72 °C for 10 min. The housekeeping genes *gyrB* and *rpoD* were also used for the phylogenetic characterization of the isolated strain. They were amplified using the primers UP1E/APrU (Yamamoto et al. 2000) and PsEG30F/PsEG790R (Mulet et al. 2009), respectively. The PCR components were as described above and PCR thermal cycling conditions were as described by Mulet et al. (2009). The amplification products for the 16S rRNA, *gyrB*, (1000 bp) and *rpoD* (700 bp) genes, were subsequently purified (Nucleospin II clean-up kit, Macherey-Nagel, Germany), cloned into the pGEM®-T easy plasmid vector system (Promega, Madison, USA) and transformed into *Escherichia coli* (DH5a competent cells, Invitrogen, USA) following standard procedures (Sambrook et al. 1989). White colonies of the transformed cells were selected for plasmid extraction (NucleoSpin Plasmid kit, Macherey-Nagel GmbH, Germany) and sequencing (Cemia S.A., Larissa, Greece).

The sequences of the 16S rRNA, *gyrB* and *rpoD* genes of the DPA-degrading bacterium and those of its closest relatives were subjected to concatenated analysis by using Mesquite ver. 2.75 (Maddison and Maddison 2011). Evolutionary distances were calculated by Jukes et al. (1969) and the dendrogram was generated by the neighbour-joining method (Saitou and Nei 1987). TREECON for Windows (Van de Peer and De Wachter 1993) was used for tree construction from distance matrix. The 16S rRNA, *gyrB* and *rpoD* gene sequences of the DPA-degrading strain selected were deposited in the GenBank under the accession numbers KJ676706–KJ676011.

Denaturing gradient gel electrophoresis (DGGE) analysis was carried out on an INGENYphorU-2×2 system (Ingeny International BV, The Netherlands). Polyacrylamide gels (8 %) in 1× TAE buffer were prepared with denaturating gradient of 45–65 % (where 100 % denaturant contains 7 M urea and 40 % formamide, AppliChem, Germany). Electrophoresis was run for 16 h at 60 °C and 75 V and gels were silver stained. Short fragments of around 190 bp containing the V3 of the 16S rRNA gene were run on the DGGE gels. They were obtained by nested PCR with primers 341f+GC-534r (Muyzer et al. 1993) using as a template the PCR products previously amplified with primers 8f-1512r. Amplification was carried out in 25 μL reactions as above and the thermocycling conditions were 95 °C for 5 min, followed by 20 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 20 s, with a final extension of 72 °C for 40 min to avoid the formation of double bands in DGGE (Janse et al. 2004). The use of an error-proof polymerase and of a low number of amplification cycles (20) minimized the possibility of PCR-driven errors in the sequences.

Clone libraries were constructed based on the PCR products obtained by primers 8f-1512r which were cloned and transformed as described above. White colonies were selected and were subjected to colony PCR using primers 341f+GC-534r. Positive clones were screened on a DGGE gel to determine their electrophoretic mobility compared with the band pattern of the original environmental sample. Representative clones for each band type were selected and plasmid DNA was extracted and sequenced as described above. The obtained sequences were edited and analysed for best match with the online tool BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Characterization of the degrading ability of the DPA-degrading isolate

In all liquid culture experiments, fresh cultures of the DPA-degrading bacterium in MSM+DPA were used as starting inocula. Briefly, fresh cultures of DPA-degrading bacteria in the mineral medium were directly established from glycerol stocks. The cultures were grown in flasks on a rotary shaker (180 rpm) at 26 °C unless stated differently. At 50 % or more DPA degradation, an aliquot of the culture was used as starting inoculum. In all cases, the initial density in the inoculated cultures ranged from 1×10^6 to 3×10^6 cfu mL^{-1} as determined by spread plating in LB.

Bacterial growth during DPA degradation

The growth kinetics of the isolated strain during DPA degradation (15 mg L^{-1}) were evaluated in both MSM and MSMN. Three inoculated replicates and two non-inoculated controls (20 mL) were prepared. The degradation of DPA and the bacterial population were determined by HPLC-UV and spread plating in LB, respectively, at the time of inoculation (T0) and 4, 8, 12, 16, 20, 24 and 28 h later.

Assessment of the capacity of the isolate to degrade high DPA concentration

The ability of the isolated strain to degrade a wide range of DPA concentrations in liquid cultures was evaluated. MSM was prepared and amended with appropriate volumes of filter-sterilized aqueous solutions of DPA (3.1, 16 and 52 g L^{-1}) prepared by the commercial formulation of DPA in order to get final pesticide concentrations in the media of 20, 120, 600, 1000 and 2000 mg L^{-1}. For every concentration examined, three inoculated cultures and two non-inoculated controls (20 mL) were prepared. DPA degradation was measured at 0, 2 and 4 days post-inoculation.

Assessment of the metabolic pathway of DPA by the isolated strain

An experiment was conducted to detect the formation of intermediate metabolites like aniline and catechol during

degradation of DPA by the isolated strain. Those two molecules have been reported as main metabolic intermediates produced during degradation of DPA by the only other known DPA-degrading strain (Shin and Spain 2009). The isolate was grown overnight in MSMN+succinate (10 mM) instead of DPA. Our hypothesis was that growing of the DPA-degrading bacterium in the absence of DPA would retard the induction of the respective catabolic genes resulting in a subsequent delay in the onset of DPA degradation enabling the more efficient determination of the formation/decay pattern of possible intermediate metabolites like aniline and catechol. The culture was centrifuged; the pellet was washed three times with sterile ddH_2O and re-suspended in MSMN to an OD_{600} of 0.15, corresponding to an inoculum density of approximately 10^6 cells mL^{-1} as determined by spread plating. Fresh cultures of MSMN+100 mg L^{-1} DPA (590 μM, prepared by the commercial formulation) were inoculated with the re-suspended pellet of the bacterium grown in MSMN+succinate. The degradation of DPA and the formation of possible metabolites were followed every 1–2 h.

In a follow-up experiment, the ability of the DPA-degrading isolate to degrade aniline and catechol was evaluated in MSMN amended with appropriate amounts of aqueous stock solutions of the studied compounds (1000 mg L^{-1}) aiming at concentrations in the final medium of 100 mg L^{-1}. Due to catechol photosensitivity, all solutions and media containing catechol were covered with aluminium foil to minimize photolytic losses. For each compound, three inoculated replicates and two non-inoculated controls (20 mL) were prepared and degradation was measured by HPLC at 0, 12 and 24 h after inoculation.

Assessment of the capacity of the isolate to degrade OPP

The ability of the isolated *Pseudomonas putida* strain to degrade the fungicide OPP was also evaluated because (a) it is used in fruit-packaging plants in a complementary way to DPA so mixtures of these two pesticides can be found in the wastewaters of a fruit-packaging plant and (b) it is a biphenylic molecule, structurally similar to DPA. Degradation was followed in MSMN amended with appropriate amounts of OPP aqueous stock solutions (100 mg L^{-1}) aiming at concentrations in the final medium of 25 mg L^{-1}. Three inoculated replicates and two non-inoculated controls (20 mL) were prepared, and degradation measurements were taken at 0, 6, 13, 20, 30 and 37 days after inoculation.

Assessment of the capacity of the isolate to degrade DPA in a range of pH and temperatures

The degrading ability of the bacterial isolate in a range of pH (4.5, 5.5, 6.5, 7.5 and 9) and temperatures (4, 15, 26 and 37 °C) was evaluated. The pH of MSM was adjusted prior to autoclaving by addition of appropriate amounts of NaOH or HCl. The pH was checked after sterilization and after growth to ensure that no significant changes have occurred during the course of the degradation study. For every pH and temperature examined, three inoculated replicates and two non-inoculated controls (25 mL) were prepared. For the pH assay, cultures were grown at 180 rpm/26 °C and degradation was daily measured. For the temperature assay, the degradation of DPA was measured at 0, 1, 2, 3, 4, 8, 11 and 30 days post-inoculation.

Assessment of the soil bioaugmentation potential of the DPA-degrading isolate

The ability of the isolated strain to degrade a range of concentrations of DPA in soil was evaluated. A clay loam soil (sand 37 %, clay 31 %, silt 32 %; pH 7.6; organic carbon content 1.05 %) with no previous history of pesticide use was collected from a field of the National Agricultural Research Foundation of Greece, Larissa. Three 500-g soil samples were treated with appropriate amounts of a filter-sterilized water stock solutions of DPA (0.2, 2 and 9 g L^{-1}) prepared by its commercial formulation, resulting in final concentrations in soil of 20, 200 and 1000 mg kg^{-1}. The soils were kept at 4 °C for 30 days to facilitate ageing of DPA residues simulating a realistic spillage situation. After this period, each soil sample was split into two portions. The first subsample was inoculated with appropriate amounts of a fresh MSM culture of the DPA-degrading bacterium to reach an inoculum level of 2×10^6 cells g^{-1} of soil as verified by LB-plate counting. The second set of subsamples received the same amount of MSM without inoculum to serve as non-inoculated controls. All soils were subsequently separated into subsamples of 20 g, which were placed into plastic bags and incubated at 25 °C. Immediately after inoculation and 2, 5, 10, 20 and 30 days thereafter, three inoculated and three non-inoculated subsamples were taken for determination of DPA degradation via HPLC analysis. Moisture content in the soil was adjusted to 40 % of the soil water holding capacity and maintained at this level.

Assessment of the degrading ability of the DPA-degrading isolate in BEM

The degrading ability of the isolated bacterium was tested in liquid BEM supplemented with 25 mg L^{-1} DPA. For comparative purposes, degradation was tested in MSM. Three inoculated cultures and two non-inoculated controls (20 mL) were prepared for every medium. Degradation was measured by HPLC at the beginning of the experiment and at 2 and 5 days post-inoculation.

Degradation data analysis

The degradation rates of DPA and of the other molecules studied were calculated in all degradation studies when more than three data points were available. In most cases, the degradation data were adequately described by the single first-order kinetics model and single degradation rates (k_{deg}) were obtained. In the cases where biphasic degradation patterns were observed, a biphasic model like the hockey-stick was used to calculate degradation rates (k_1 and k_2 for the two phases of the degradation pattern). The χ^2 test (<15 %), visual inspection and the distribution of the residuals were used as criteria to assess the goodness of fit. Details about the kinetic models used could be found elsewhere (FOCUS 2006). All calculations were performed with the statistical package R 3.0.2v.

Results and discussion

Isolation and identification of a DPA-degrading bacterium

In both MSM and MSMN, a rapid degradation of DPA was observed from the first enrichment cycle, with complete elimination of DPA within 2 days (Supplementary data Fig. 1). After plating of the fourth enrichment cycle cultures, a total of 40 growing colonies were selected and assayed for DPA degradation in the corresponding liquid media. Among them, 14 were able to completely degrade DPA within 5 days. Upon plating, all isolates appeared to be composed of the same single morphotype. Two isolates per medium, named M1 and M3 from MSM and N4 and N6 from MSMN, were randomly selected and their purity was assessed via PCR-DGGE targeting the 16S rRNA gene. All isolates showed the same fingerprint pattern comprising four main bands (Supplementary data Fig. 2a). These results challenged our initial conclusion about the visual purity of the isolates obtained. In order to clarify this, 21 colonies from the isolates M1 and N4 growing on MSM and MSMN+DPA plates, respectively, were subjected to colony PCR and DGGE screening. All colonies gave a DGGE profile identical to the initial DPA-degrading cultures composed of the same four dominant bands (Supplementary data Fig. 2b).

Based on the abovementioned results, we hypothesized that all isolates constituted the same bacterium showing an inherent sequence polymorphism in the V3 region of the 16S rRNA gene. The number of copies of rRNA operons per bacterial genome could vary from 1 to 15 (Andersson et al. 1995), and in several cases, sequence polymorphisms between those copies have been observed (Wang et al. 1997). The almost full sequence of the 16S rRNA gene of the isolate N4 was analysed via clone libraries. All selected clones showed high sequence match (>99 %) to *P. putida* strains. Analysis of the sequences of the V3 region of the clones obtained showed base polymorphism in six base positions (Table 1) whose different combinations resulted in the formation of four different sequences, in line with the appearance of four bands in the DGGE fingerprint of the isolate (Supplementary data Figs. 2a and 2b). Sequence polymorphism of the 16S rRNA gene is an intrinsic characteristic of the genus *Pseudomonas* (Stover et al. 2000; Nelson et al. 2002) with *P. putida* strains possessing 6 to 7 copies of the 16S rRNA gene (Bodilis et al. 2012).

Despite of the general use of 16S rRNA gene for the phylogenetic characterization of bacteria, its resolution at the species level is low for *Pseudomonas* (Yamamoto et al. 2000; Anzai et al. 2000). Thus, the use of housekeeping genes like *gyrB* and *rpoD* in combination with the 16S rRNA gene has been proposed to increase the resolution of phylogenetic analysis of pseudomonads at the species level (Yamamoto et al. 2000; Mulet et al. 2009).

The bacterial strain phylogeny was constructed using the concatenated alignment of 16S rRNA, *gyrB* and *rpoD* gene amplicons (3096 bp) derived from isolates N4 and M1. For the 16S rRNA gene of isolates N4 and M1 where sequence polymorphisms were observed, the consensus sequence for its isolate was used for phylogenetic analysis. The sequences of the three housekeeping genes of the two isolates (N1 and M1) were identical verifying that they corresponded to the same strain. The phylogeny output suggested the assignment of the DPA-degrading strain within the *P. putida* group as defined by Mulet et al. (2009) with the closest relatives being several *P. putida* strains known to be involved in the degradation of organic pollutants (Fig. 1). *P. putida* strain ND6 was isolated from industrial wastewater for its ability to degrade naphthalene (Zhang et al. 2000); *P. putida* strain F1 is one of the best studied toluene-degrading bacteria (Wackett and Gibson 1988), while *P. putida* KT2440 was able to degrade a range of natural and xenobiotic aromatic compounds through different catabolic pathways (Jimenez et al. 2002; Nelson et al. 2002). The isolate was named *P. putida* strain DPA1 and it was used in all further experiments.

Characterization of the bacterial degrading ability

Bacterial growth during DPA degradation

In MSM and MSMN, the degradation of DPA was biphasic with an initial lag phase (k_1=0.014 and 0.005 h^{-1}, respectively) followed by a rapid degradation phase (k_2=0.850 and 0.250 h^{-1}, respectively). The latter phase coincided with a stoichiometric growth of the DPA-degrading bacterium which peaked (5.6–6.5×10^7 cells mL^{-1}) at 24 h when DPA degradation was completed (Fig. 2). In both media, the observed degradation and bacterial growth kinetics indicate that the isolated strain was able to degrade and utilize DPA as the sole C and N

Table 1 Analysis of the 200-bp fragments corresponding to the V3 region of the 16S rRNA gene of the DPA-degrading culture N4

Clones	Position (bp) in sequence according to the *Escherichia coli* 16S rRNA gene numbering						
N4	Primer 341f	416	464	514	515	517	Primer 534r
A-1	341-357	A	T	C	T	T	518-534
B-2	341-357	*G*	T	C	T	T	518-534
C-3	341-357	A	*C*	*A*	*A*	*C*	518-534
D-1	341-357	A	T	C	T	T	518-534
E-4	341-357	A	*C*	C	T	T	518-534

The bases showing sequence polymorphisms in the different clones are shown in italics. The positions of the forward (Muyzer F) and reverse (Muyzer R) primers in each clone sequence are also indicated. The numbers following the clones A to E indicate the four copies of the 16S rRNA gene showing sequence polymorphism in the V3 region

source resulting in growth yields of 1.8×10^8 and 1.9×10^8 cells mg^{-1} DPA in MSM and MSMN, respectively, which are comparable with the yield coefficient of 1.6×10^8 cells mg^{-1} toluene reported by Shreve and Vogel (1993) for the toluene-degrading *Pseudomonas* strain K3-2.

Degradation of increasing concentrations of DPA by the isolated strain

P. putida strain DPA1 was able to completely degrade up to 600 mg L^{-1} of DPA within 2 days and achieved ca. 85 %

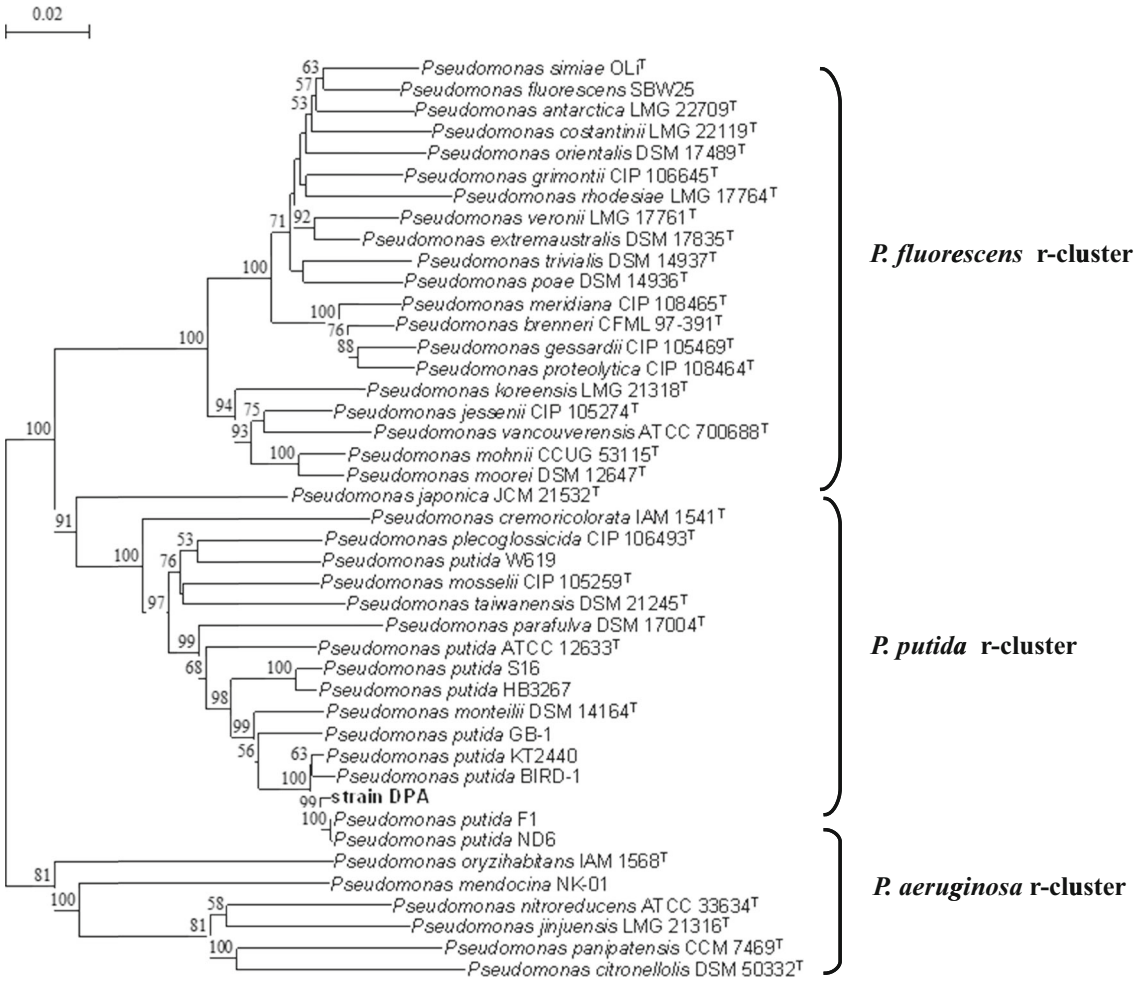

Fig. 1 Phylogenetic position of the selected DPA-degrading bacterium based on concatenated analysis of the 16S rRNA, *gyrB* and *rpoD* gene sequences (3096 bp in total). Distance matrix was calculated by Jukes et al. (1969) and the dendrogram was constructed by the neighbour-joining method (Saitou and Nei 1987). *Numbers on the nodes* denote percent bootstrap values based on 1000 replicates (less than 50 % bootstrap support is not shown). *Scale bar* represents 0.02 substitutions per site. The three well-supported clusters in the 16S rRNA phylogeny (*P. fluorescens*, *P. putida* and *P. aeruginosa*), as described by Bodilis et al. (2012), are indicated

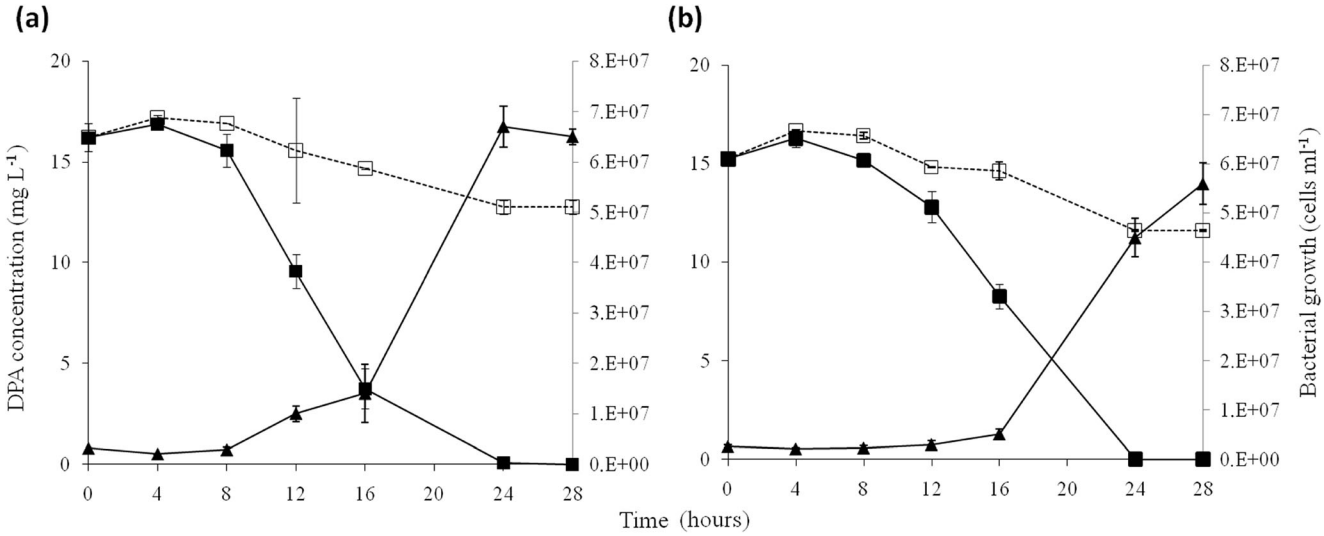

Fig. 2 Degradation of DPA (15 mg L^{-1}, chemical standard) (*black square*) by *P. putida* strain DPA1 and its concurrent growth (*black triangle*) in MSMN (**a**) and MSM (**b**). DPA degradation in non-inoculated controls is also shown (*white square*, *dotted line*). *Error bars* represent the standard deviation of the mean

degradation of the 1000 mg L^{-1} concentration in the same time (Supplementary data Fig. 3). In addition, the DPA-degrading strain was able to degrade the highest concentration tested, 2000 mg L^{-1}, within 4 days. The degradation of DPA in the non-inoculated controls was negligible, verifying the high degrading capacity of our isolate against particularly high DPA concentrations which might be encountered if applied for the biodepuration of DPA-containing wastewaters. This is the first report of a bacterium able to metabolize rapidly such high levels of DPA. In previous studies, DPA-degrading bacteria were reported to completely degrade up to 16 mg L^{-1} of DPA in more than 12 days (Christodoulatos et al. 1997) or up to 40 mg L^{-1} within 2 days (Shin and Spain 2009), although tests with higher concentrations were not reported in those studies.

Assessment of the metabolic pathway of DPA by the isolated P. putida strain

The degradation of DPA by *P. putida* strain DPA1 showed a biphasic pattern with an initial lag phase (k_1=0.022 h^{-1}) followed by a rapid degradation phase (k_2=0.510 h^{-1}) which coincided with the transient formation of low amounts of aniline and catechol (Fig. 3). Negligible degradation of DPA was observed in the non-inoculated control, and traces of aniline and catechol were also detected at later time points. The low amounts of those metabolites detected might be the result of the parallel operation of formation–degradation mechanisms in the isolated bacterium which prevent the accumulation of any of these two intermediates. The transient formation of these two intermediates during degradation of DPA by the *P. putida* strain DPA1 is in agreement with previous observations by Shin and Spain (2009) who also noted their temporary formation in cultures of *Burkholderia* sp. JS667.

When the ability of *P. putida* strain DPA1 to degrade aniline and catechol was assessed, both compounds were completely degraded within 14 h with biphasic degradation rates of k_1=0.051 and k_2=9.36 h^{-1} for aniline and k_1=0.056 and k_2=0.73 h^{-1} for catechol. In contrast, negligible degradation of DPA in the non-inoculated controls was observed (data not shown). These data coupled with the transient formation of aniline and catechol during the degradation of DPA provide strong evidence for the operation of a pathway in *P. putida* strain DPA1 similar to the one reported by Shin and Spain (2009) in a *Burkholderia* sp.

Assessment of the capacity of the isolate to degrade the fungicide OPP

The DPA-degrading isolate was able to degrade the structurally similar fungicide OPP but at a substantially lower rate, with more than 80 % degradation observed in 37 days (k_{deg}= 0.055 day^{-1}) compared to its negligible degradation in the abiotic control (Fig. 4). The low degradation capacity of the DPA-degrading bacterium against OPP could be of co-metabolic nature and could be linked with the activity of monooxygenases or dioxygenases with relaxed substrate specificity that could attack one of the two aromatic rings adding one or two atoms of oxygen then converted by dehydrogenases in hydroxylated groups (Suenaga et al. 2009). The capacity of the *P. putida* strain DPA1 to degrade, even at a slower rate, the fungicide OPP which is also used in the fruit-packaging industry and its residues could co-occur with DPA in the wastewaters constituting a desirable asset for its

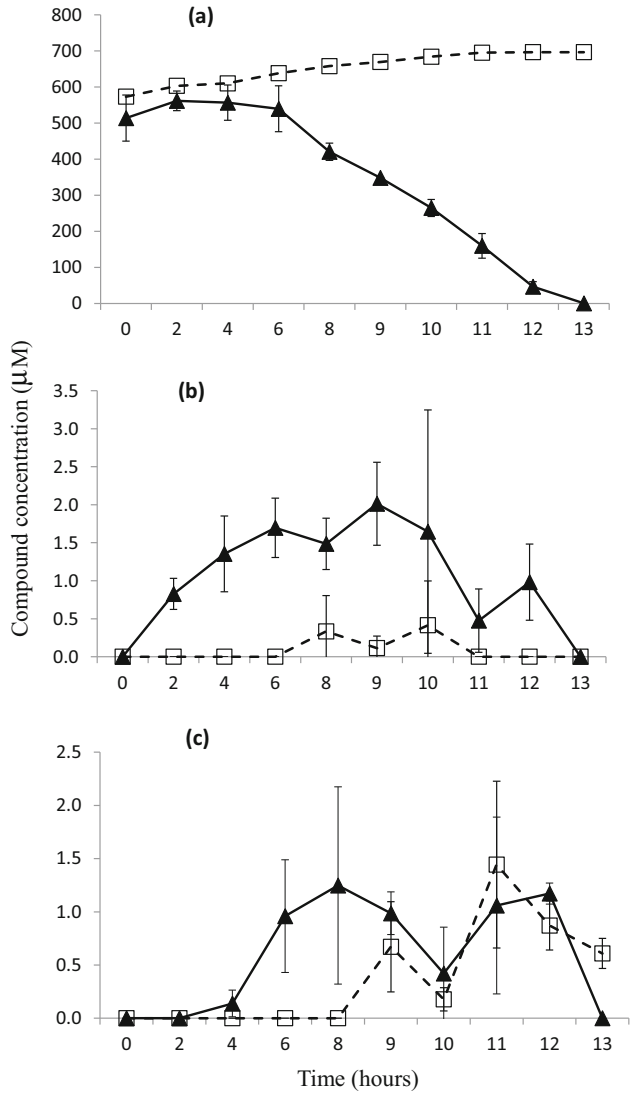

Fig. 3 Degradation of 590 μM DPA (commercial formulation) (**a**) and the formation of aniline (**b**) and catechol (**c**) in MSMN inoculated (*black triangle*) or not inoculated (*white square*, *dashed line*) with *P. putida* strain DPA1. *Error bars* represent the standard deviation of the mean

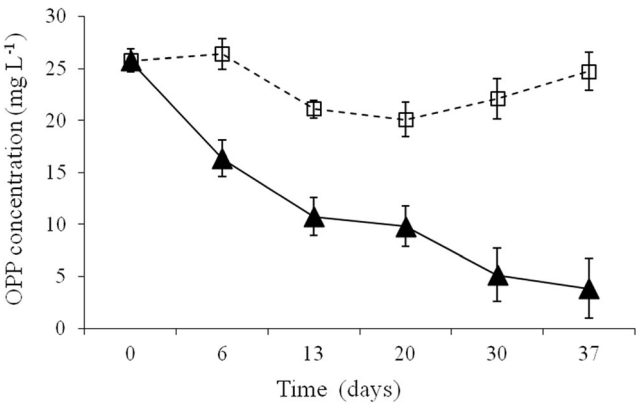

Fig. 4 Degradation of OPP (25 mg L^{-1}, chemical standard) by *P. putida* strain DPA1 selected (*black triangle*). Degradation of all tested molecules in non-inoculated controls (*white square*, *dashed line*) is also presented. *Error bars* represent the standard deviation of the mean

practical application in the treatment of wastewaters from fruit-packaging plants.

The degradation of DPA by the isolated strain in a range of pH and temperatures

pH and temperature are among the main factors influencing the degradation ability of inoculants in full-scale applications (Karpouzas and Walker 2000; Awasthi et al. 2000), and optimization of such conditions is crucial for the efficiency of the process. For this purpose, the effect of pH and temperature were evaluated. *P. putida* strain DPA1 was able to rapidly degrade DPA independently of the pH of the medium with almost complete degradation occurring within a day in all pH tested (4.5 to 9) (data not shown). No DPA degradation was observed in the non-inoculated samples at all pH values tested.

When the degrading ability of the bacterium was tested at various temperatures, differences in the degradation of DPA were observed (Fig. 5). The most rapid degradation was observed at 26 °C where complete degradation of DPA was evident in 1 day. In contrast, relatively slower degradation was observed at 15 and 37 °C where complete or nearly complete degradation of DPA was measured at 3 days. At 4 °C, similar degradation rates in the inoculated and the non-inoculated cultures were observed (k_{deg_inoc}=0.28 day^{-1} vs $k_{deg\_control}$=0.24 day^{-1}), suggesting inhibition of the biotic degradation of DPA at this temperature. The considerable degradation of DPA observed in the non-inoculated control at 4 °C could be attributed to abiotic degradation processes since plating of these samples onto LB and MSMN+DPA agar plates did not show any bacterial growth.

Thus, *P. putida* strain DPA1 was able to rapidly degrade DPA in a wide range of pH (4 to 9) and temperatures (mostly mesophilic), making it suitable for bioremediation applications.

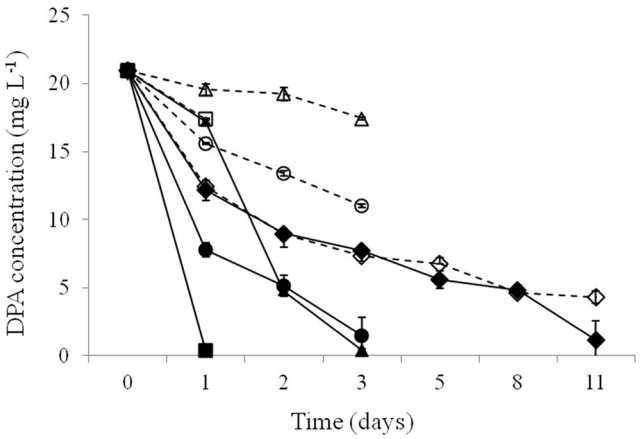

Fig. 5 Degradation of DPA (20 mg L^{-1}, chemical standard) by *P. putida* strain DPA1 at 4 °C (*black diamond*, *white diamond*), 15 °C (*black triangle*, *white triangle*), 26 °C (*black square*) and 37 °C (*black circle*, *white circle*) (*closed symbols*, *solid lines*). Degradation in corresponding non-inoculated controls is also presented (*empty symbols*, *dotted lines*)

Regarding previously reported DPA-degrading bacteria, no data are available regarding their pH and temperature tolerance (Christodoulatos et al. 1997; Shin and Spain 2009).

Assessment of the soil bioaugmentation potential of the DPA-degrading strain

The potential use of *P. putida* strain DPA1 for the decontamination of such soils was assessed by testing its degrading ability in an artificially contaminated soil at three concentrations levels: 20, 200 and 1000 mg kg^{-1}. Inoculated soil showed substantially higher dissipation rates of DPA (k_{deg_20}= 0.53 day^{-1}, k_{deg_200}=0.53 day^{-1} and k_{deg_1000}=0.60 day^{-1}) relatively to the corresponding non-inoculated samples (k_{deg_20}= 0.092 day^{-1}, k_{deg_200}=0.060 day^{-1} and k_{deg_1000}=0.070 day^{-1}) (Fig. 6). At all concentrations examined, DPA was almost completely dissipated (>92 %) within 5 days in the inoculated samples compared to the corresponding controls where only 10, 41 and 43 % dissipation was measured in the samples amended with 20, 200 and 1000 mg kg^{-1} DPA, respectively. After this rapid phase, dissipation rates declined, probably due to the limited bioavailability of the pesticide (Cullington and Walker 1999). In the non-inoculated soil samples, DPA showed a moderate persistence with less than 15 % of the initial DPA remaining in the soils amended with the different dose rates after 30 days of incubation. Our data suggest that *P. putida* strain DPA1 has high bioaugmentation potential and it is able to dissipate particularly high concentration of DPA in soil (1000 mg kg^{-1}) from wastewater disposal sites.

Degradation of DPA by the isolated strain in BEM

Previous studies with fungicides used in citrus fruit-packaging plants (DPA was not included) showed that biobed systems could be used, in a modified form, for the treatment of wastewater from small- and medium-sized fruit-packaging plants producing low wastewater volumes (Omirou et al. 2012). The depuration efficiency of such systems could be implemented by bioaugmentation with microorganisms known to be able to degrade the contaminants of interest (Karanasios et al. 2012). Thus, the potential application of the isolated bacterium in biobed-like systems was investigated by determining its degradation capacity in the liquid medium BEM whose nutritional composition resembled as much as possible those of biomixtures used for the packing of on-farm biobed systems (Castillo et al. 2008). *P. putida* strain DPA1 degraded DPA in BEM and MSM at similar rates (data not shown) with almost complete degradation observed in 2 days. These results provide initial evidence for the adaptability of the *P. putida* strain DPA1 to biobed conditions and its potential for future implementation in the bioaugmentation of biobeds receiving DPA-containing wastewater.

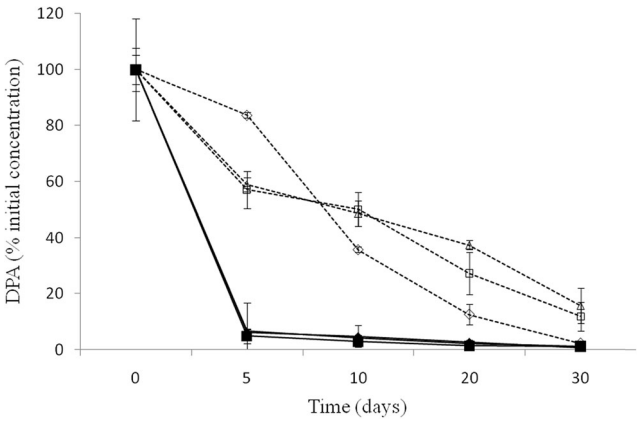

Fig. 6 Dissipation of 20 (*black diamond*, *white diamond*), 200 (*black triangle*, *white triangle*) and 1000 mg kg^{-1} (*black square*, *white square*) of DPA (commercial formulation) in soil inoculated (*closed symbols*, *solid lines*) or non-inoculated (*empty symbols*, *dotted lines*) with *P. putida* strain DPA1. *Error bars* represent the standard deviation of the mean

Conclusions

In this study, a particularly effective DPA-degrading strain that could utilize DPA as the sole C and N source was isolated and further identified via molecular-based analysis as *P. putida* strain DPA1. This isolate degraded DPA via the production of aniline and catechol which were further transformed. *P. putida* strain DPA1 showed capacity to degrade (a) up to 2000 mg L^{-1} of DPA in liquid medium and 1000 mg kg^{-1} of DPA when inoculated in soil, (b) DPA under a range of pH and temperatures, (c) the structurally similar fungicide OPP and (d) DPA in media simulating the nutritional conditions of biobed packing substrates. All these provide strong evidence for its high potential for future biotechnological applications, including the in situ decontamination of DPA-polluted wastewater disposal sites, the bioaugmentation of biobed systems receiving DPA-contaminated wastewaters and its use as tailored-made inocula in biofiltration systems devoted to the treatment of wastewaters from the fruit-packaging industry. Ongoing genomic and proteomic analysis of *P. putida* strain DPA1 will shed light into the genes/enzymes involved in the metabolism of DPA by this isolate.

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