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RESEARCH ARTICLE

Proteomic examination of polyester-polyurethane degradation by *Streptomyces* sp. PU10: Diverting polyurethane intermediates to secondary metabolite production

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Abstract

Global plastic waste accumulation has become omnipresent in public discourse and the focus of scientific research. Ranking as the sixth most produced polymer globally, polyurethanes (PU) significantly contribute to plastic waste and environmental pollution due to the toxicity of their building blocks, such as diisocyanates. In this study, the effects of PU on soil microbial communities over 18 months were monitored revealing that it had marginal effects on microbial diversity. However, *Streptomyces* sp. PU10, isolated from this PU-contaminated soil, proved exceptional in the degradation of a soluble polyester-PU (Impranil) across a range of temperatures with over 96% degradation of 10 g/L in 48 h. Proteins involved in PU degradation and metabolic changes occurring in this strain with Impranil as the sole carbon source were further investigated employing quantitative proteomics. The proposed degradation mechanism implicated the action of three enzymes: a polyester-degrading esterase, a urethane bond-degrading amidase and an oxidoreductase. Furthermore, proteome data revealed that PU degradation intermediates were incorporated into *Streptomyces* sp. PU10 metabolism via the fatty acid degradation pathway and subsequently channelled to polyketide biosynthesis. Most notably, the production of the tri-pyrrole undecylprodigiosin was confirmed paving the way for establishing PU upcycling strategies to bioactive metabolites using *Streptomyces* strains.

INTRODUCTION

Polyurethanes (PU) comprise a diverse family of polymers formed by reacting diisocyanates and polyols (Liu et al., 2021). Their mechanical, thermal and various other properties can vary extensively depending on the specific monomers employed; however, they all share the characteristic urethane (carbamate) bond within their backbone. PUs often feature additional bonds, such as ester and ether bonds, depending on the choice of polyol used in their synthesis which greatly impacts their properties and biodegradability (Jin et al., 2022). Due to their versatility, polyurethanes have found application in a wide array of industries, including building and construction, automotive manufacturing, footwear, mattresses and more (Liang et al., 2021). Recently, there has been a growing apprehension regarding plastic pollution on

a global scale. Notably, PUs have emerged as one of the most toxic polymers releasing carcinogenic and ecotoxic compounds with adverse environmental impacts (Yuan et al., 2022; Zimmermann et al., 2019). This is primarily attributed to the additives used in the production of PUs. Furthermore, the most common monomers employed in their manufacture, namely, 4,4′-methylenediphenyl diisocyanate (4,4′-MDI) and toluene diisocyanate (TDI), are classified as carcinogenic substances as well (Luft et al., 2017). On a global scale, annual plastic production exceeds 380 million metric tons, with polyurethanes ranking as the sixth most prevalent polymer, accounting for 5.5% of global production trailing slightly behind polyethylene terephthalate (PET), which accounts for 6.2% of total plastics produced (PlasticsEurope, 2022). Unlike polyethylene, polypropylene and polyvinyl chloride which possess an unreactive C-C backbone, both PU and PET consist of hydrolyzable bonds, making them more susceptible to chemical and enzymatic depolymerization. This vulnerability to degradation has already been successfully harnessed in the case of PET (Tournier et al., 2020) but an effective means of PU depolymerization remain elusive.

Different chemical and biological methods for PU degradation and recycling have been employed with varying success rates. Currently, chemical methods such as alcoholysis, hydrolysis, aminolysis and phosphorolysis are primarily used for the degradation of PU materials with glycolysis standing out as the most widely used and effective, even being employed on an industrial scale (Simón et al., 2018). Despite numerous approaches, the energy-intense conditions required and associated environmental drawbacks have restricted the practical applications of chemical recycling. Furthermore, the intricate nature of PUs hampers the development of a 'one size fits all' solution with depolymerization efforts yielding complex mixtures of monomers and oligomers unsuitable for repolymerization (Utomo et al., 2020). Accordingly, biological degradation has emerged as a more suitable and environmentally friendly route for PU degradation.

Plastic-polluted soil and landfills have proved an invaluable reservoir of PU-degrading microbial communities predominately comprised of *Bacillus* and *Pseudomonas* species (Park et al., 2023; Vargas-Suárez et al., 2019, 2021), along with fungal strains (Magnin, Hoornaert, et al., 2019). Numerous PU-degrading strains have been identified (Gambarini et al., 2021; Magnin et al., 2020); however, identifying the enzymes involved in PU degradation has proved challenging. To date, 26 PU-degrading enzymes from microorganisms belonging to diverse habitats have been identified and characterized primarily cutinases, esterases and lipases (Buchholz et al., 2022). The urethane bond has proved exceedingly resilient to enzymatic attack (Pantelic et al., 2023) and most of the degradation studies were thus performed on polyester-PU and the subsequent degradation was attributed primarily to ester bond hydrolysis (Di Bisceglie et al., 2022). In contrast to esterases and cutinases, amidases have been found to preferably hydrolyse the urethane bond and have been used both to degrade small urethane bond-containing molecules (Branson et al., 2023) and to degrade thermoplastic PU in combination with esterases (Magnin, Hoornaert, et al., 2019). Due to a lack of efficient degradation strategies, investigation into PU upcycling has been limited with chemical upcycling strategies leading the way (Liu, Zeng, et al., 2023; Morado et al., 2023). Alternatively, the only report on biological upcycling describes the transformation of selected PU monomers to rhamnolipids by a synthetic microbial consortium (Utomo et al., 2020).

As integral parts of the terrestrial microbiome *Streptomyces* strains have been under-represented in plastic degradation studies with scattered reports on the degradation of polyolefins (El-Shafei et al., 1998; Soud, 2019), polyhydroxyalkanoates (Gangoiti et al., 2012) and one identified enzyme-SM14est reported to efficiently degrade PET (Carr et al., 2023). Notably, as of our current knowledge, despite their renowned status as the most prolific antibiotic producers (Shepherdson et al., 2023) no *Streptomyces* strains have been employed for the upcycling of plastic waste or plastic-related monomers.

In this work, we report the first comprehensive quantitative proteomics analysis of proteins involved in PU degradation, PU degradation intermediate metabolic pathways and the link between primary and secondary metabolism in the newly isolated *Streptomyces* sp. PU10. Additionally, we monitored the effect of PU waste on the composition of a soil microbial community via 16S rDNA community analysis and isolated several highly active *Streptomyces* strains.

EXPERIMENTAL PROCEDURES

Soil sample preparation

A composite model compost (original soil) was prepared by thoroughly mixing equal amounts of commercial gardening compost soil, soil from a vineyard (Belgrade, Serbia, 44.611443, 20.563120) and soil obtained from a field (Ruma, Serbia, 44.979366, 19.828713). This composite soil (5 kg) was partitioned into two separate polypropylene containers. One container held the unmodified composite soil (C soil), while the other contained composite soil enriched with 200 g of PU construction waste material (PU soil). The soil samples were incubated for 18 months, at 25°C with 1 L of sterilized water being added every 4 weeks to maintain 100% humidity.

Microorganism isolation and identification

Soil sampling (approximately 1 g) was conducted from each container at three distinct positions (10 cm from the three different edges of the container at a depth of 5 cm) and pooled. Samples were suspended in sterile PBS (phosphate-buffered saline, 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.245 g/L NaH_2PO_4) and homogenized via vigorous shaking for 20 min. The test tubes were then left undisturbed for 10 min for the larger particles to settle and serial dilutions were prepared.

The isolation process was executed through inoculation of agar plates with complex media favouring the isolation of diverse sporulating (MSF—Mannitol Soy Flower, 20 g/L soy flower, 20 g/L mannitol and 20 g/L agar) and non-sporulating (LA—Luria Bertani agar, 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl and 15 g/L agar) microorganisms (Narancic et al., 2012). After inoculation with 100 μL of serial dilutions in triplicate, the plates were incubated at 30°C for 3 days. Morphologically distinct colonies were then selected and subcultured onto fresh plates. The quantification of colony-forming units (CFUs) was performed using 1 g of soil, and total DNA was extracted for 16S community analysis. Alternatively, MSM (Mineral Salt Medium, 9 g/L $Na_2HPO_4 \times 12H_2O$, 1.5 g/L KH_2PO_4, 1 g/L NH_4Cl, 0.2 g/L $MgSO_4 \times 7H_2O$, 0.2 g/L $CaCl_2 \times 2H_2O$, 0.1% trace elements solution, 0.025% N-Z amine and 15 g/L agar) containing 0.5% (v/v) Impranil DLN SD (Covestro, Leverkusen, Germany) (IMP plates) (Molitor et al., 2020) was inoculated and followed by a 14-day incubation at 30°C. The appearance of clearing halos on these plates signified the presence of PU-degrading activity. The quantification of halo-forming CFUs was carried out and halo-forming colonies were plated onto fresh plates.

Isolated strains of interest were identified by 16S rDNA sequencing using standard 1492R (5′ TACGGYTACCTTGTTACGACTT 3′) and 27F (5′ AGAGTTTGATCMTGGCTCAG 3′) primers. The 16S rDNA was amplified via PCR (FastGene TAQ PCR Kit, Nippon Genetics, Düren, Germany) and sequenced by Macrogen Europe BV, Netherlands.

Evaluating Impranil degradation

Ten millilitres of MSM medium containing 0.5%–1.5% (v/v) Impranil as sole carbon and energy source was inoculated with 0.1% (v/v) overnight cultures grown in LB medium and incubated for 24–72 h at 25–42°C and 180 rpm. Cultures were centrifuged at 5000 *g* for 15 min (Eppendorf 5804 centrifuge, Hamburg, Germany) and the supernatant was filtered through a 0.45 μm syringe filter after which turbidity was assessed spectrophotometrically (Epoch Microplate Spectrometer, BioTek, Winooski, VT, USA) at 600 nm to assess Impranil degradation (Zhang et al., 2022). The percentage of Impranil degradation was calculated comparing the culture to control supernatants. Culture supernatants were freeze-dried using an Alpha 1–2 LD plus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) to be used for Fourier-transform infrared spectroscopy (FTIR) analysis. FTIR spectra were obtained using an IR-Affinity spectrophotometer (NICOLET iS10; Thermo Fisher Scientific, Waltham, MA, USA) in attenuated total reflection mode. The measurements were conducted within the specified wavenumber range of 4000–400 cm^{-1}, at room temperature, with a resolution of 4 cm^{-1}. The fixed number of scans was set at 32. Additionally, filtered culture supernatants of *Streptomyces* sp. PU10 grown for 48 h at 37°C and 180 rpm in MSM medium containing 1% glucose and 0.1% Impranil (v/v) (used to elicit Impranil-degrading enzymes) were used for clearing assays to identify possible degradation products via LC–MS (Supplementary Material S1).

16S rDNA community analysis

All colonies grown on LA, MSF and IMP plates were collected using sterile swabs, suspended in PBS buffer and total DNA from the colonies was extracted using ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA, USA). These samples represented the cultivable microbial strains out of the overall diversity. Using the same kit, DNA was extracted directly from 250 mg of the Original (before incubation, and kept at −20°C), C and PU soil representing the total community diversity.

Sequencing of full-length 16S rDNA was conducted using the MinION next-generation sequencing, featuring a Flongle adapter and R9.4.1 flow cell (Oxford Nanopore Technology, UK). Employing the 16S barcoding kit 1–24 and associated protocol, DNA libraries were prepared for Nanopore sequencing. The input comprised 40 ng of DNA per sample, and 35 amplification cycles were administered to enhance barcoding PCR sensitivity. Equal proportions of each barcoded sample constituted the library, which was sequenced over 30 h. Following base calling using Guppy (v6.4.2) in high accuracy mode, reads were subjected to length filtration (from 1200 to 1800 base pairs). The quality of the reads was assessed, and sequencing adapters were trimmed using Porechop (v0.2.4). Taxonomic classification was achieved using KrakenUniq (Breitwieser et al., 2018; Odom et al., 2023) in conjunction with the KrakenUniqanti database. Shannon's diversity index, inverse Simpson's index and Bray–Curtis dissimilarity index were calculated using Krakentools (Lu et al., 2022). The visualization of taxonomic data was realized using Pavian (Breitwieser & Salzberg, 2020) in tandem with in-house analysis pipelines (MicroLife Solutions, the Netherlands).

Genome sequencing

Whole genome sequencing was performed using MinION (Oxford Nanopore Technologies, Oxford, UK). From isolated genomic DNA, a library was constructed using an LSK-114 kit after which the library was sequenced on a single Flongle R10.4 flow cell (Oxford Nanopore Technologies, Oxford, UK). Base calling was done in Dorado (v0.3.4) using the super-accuracy setting. Genome quality was assessed using Benchmarking Universal Single-Copy Orthologs (BUSCO 5.2.2) (Manni et al., 2021) and annotated using PGAP (Li et al., 2021), InterProScan (v5.65–97.0) (Jones et al., 2014) was used to gain further information on the predicted proteins, signal peptides were predicted using SignalP6 (Teufel et al., 2022), biosynthetic gene clusters were identified using antiSMASH 7.0.0 (Blin et al., 2023), and proteins were assigned to KEGG metabolic pathways using BlastKoala (Kanehisa et al., 2016). Finally, potential plastic-degrading proteins were identified by Blasting against the Plastics-Active Enzymes (PAZy) database (Buchholz et al., 2022).

Sample preparation and LC–MS/MS analysis

For proteomic investigations, the variations in the protein make-up of *Streptomyces* sp. PU10 when grown using Impranil or glucose as the carbon source was compared. Cultures were incubated in 50 mL of MSM medium, containing 1% Impranil or 1% glucose, for 72 h at 30°C and 180 rpm in triplicate. Subsequently, cells were harvested through centrifugation at 5000 *g*, 4°C, for 20 min (Eppendorf 5804 centrifuge, Hamburg, Germany). The supernatant (extracellular protein fraction) was collected, and the cell pellet was treated with BugBuster® Protein Extraction Reagent (Merck KGaA, Darmstadt, Germany) and sonicated (Soniprep 150, MSE Ltd., Lindon, UK) for 10 min at 10 kHz. The cell debris was removed by centrifugation at 14,000 *g* for 20 min (Eppendorf Centrifuge 5417 R, Hamburg, Germany) and the supernatant was collected (intracellular protein fraction). Both protein fractions were dialysed against 20 mM Tris–HCl buffer (pH 7) using SpectraPor® Dialysis Tubing (Spectrum Laboratories, Inc., Rancho Dominguez, USA) with a 3.5 kDa cut off. The protein extracts (extracellular in triplicate and intracellular in duplicate) were freeze-dried and analysed via LC–MS/MS by VIB Proteomics Core (Gent, Belgium) detailed in the Supporting Material S1.

Differential protein abundance was tested using the empirical Bayes approach with the imputation of missing values as implemented in prolfqua (Smyth, 2004). The mass spectrometry proteomics data along with an extended description of the experimental procedure and the quality control reports have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al., 2022), with the data set identifier PXD047534 and 10.6019/PXD047534. The enrichment of differentially abundant proteins was tested with Fisher's exact test and multiple test correction was performed with Benjamini–Hochberg at 5% FDR. The population set was set to the reliably quantified proteins of the respective fraction. Enrichment of gene ontology (GO) terms was tested using goatools v1.3.1 (Klopfenstein et al., 2018) with the go-basic.obo release 2023-04-01. GO terms were derived from the PGAP and Interproscan annotations. Enrichment of KEGG pathways was tested with KOBAS v3.0.3 (Bu et al., 2021) and annotations were derived using the *Streptomyces* sp. CCM_MD2014 KEGG genome.

Undecylprodigiosin extraction and detection

After 7 days of incubation at 30°C in MSM medium with 5 g/L of Impranil as sole carbon source, *Streptomyces* sp. PU10 cells were harvested by centrifugation at 5000 *g*, 4°C, for 20 min (Eppendorf 5804 centrifuge, Hamburg, Germany) and freeze-dried using an Alpha 1–2 LD plus freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). To the dried cell pellet, 5 mL of methanol with 1% (v/v) HCl was added and incubated for 3 h at 180 rpm. The presence of undecylprodigiosin was confirmed by the UV–Vis spectrum and the characteristic absorption maximum at 533 nm using a UV-1900i UV–Vis spectrophotometer (Shimadzu, Kyoto, Japan) and the concentration was approximated using a standard curve (Liu, Fang, et al., 2023). Additionally, the presence of undecylprodigiosin was investigated via LC–MS in single ion monitoring (SIM) mode at 394.27 m/z (using the same method as described in the Supplementary information for the detection of possible Impranil degradation products).

RESULTS AND DISCUSSION

Assessment of PU enrichment on microbial community and isolation of Impranil-degrading strains

The introduction of PU waste had little impact on the overall number and diversity of microorganisms when incubated in the model soil for 18 months (Figure S1A,B). 16S rDNA community analysis yielded 43,108, 49,180 and 45,644 full-length classified reads for the Original, C and PU soil respectively. The most abundant genera among all samples were *Bacillus* accounting for 6%–12% of the reads followed by *Lysobacter*, *Woeseia*, *Mesorhizobium*, *Haliangeum* and *Lutenimonas* with

no clear grouping. Notably, *Hyphomicrobium* species, recently associated with PU-degrading communities (Park et al., 2023), made up a substantial portion of the community with between 2.2% and 3.3% of reads. To get a context of the overall diversity changes in the samples, statistically significant metrics Shannon's diversity index and inverse Simpson's index were used. The alpha diversity of samples was relatively uniform between the control and PU-enriched samples (Figure 1A,B). Notably, the diversity in both C and PU soil was lower than in the original soil demonstrating the incubation conditions had a more pronounced effect on microbial diversity than the PU enrichment. Explained by the lack of changing environmental factors, constant temperature and humidity favoured microorganisms best suited for these exact conditions which ultimately lead to an overall decrease of diversity in comparison to the fresh soil sample.

To determine more nuanced community responses to PU waste changes in abundance of reported PU degrading strains were investigated. For this, a list containing 24 genera previously reported to degrade PU was compiled from multiple sources (Gambarini et al., 2021, 2022; Magnin et al., 2020). In all soil samples, *Bacillus* was the dominant PU-degrading genera comprising 57%–77% out of all reads assigned to PU-degrading genera (Figure 1D). Out of the 24 genera assessed, only *Alicycliphilus*, *Chryseobacterium* and *Micrococcus* were not detected in any samples. Although most of the PU-degrading genera were ubiquitous among samples and with marginal changes in abundance the total number of reads attributed to PU-degraders rose from 11.4% in original soil to 15.7% and 16.9% in C and PU soil respectively. The rise in abundance within both C and PU soil could potentially be attributed to specific humidity conditions maintained during the incubation period rather than the effect of PU waste. Overall, based on these results PU enrichment had no impact on the abundance and diversity of microbial community over the time course examined in this study. Similar studies have reported significantly lowered alpha diversity metrics in soil contaminated with

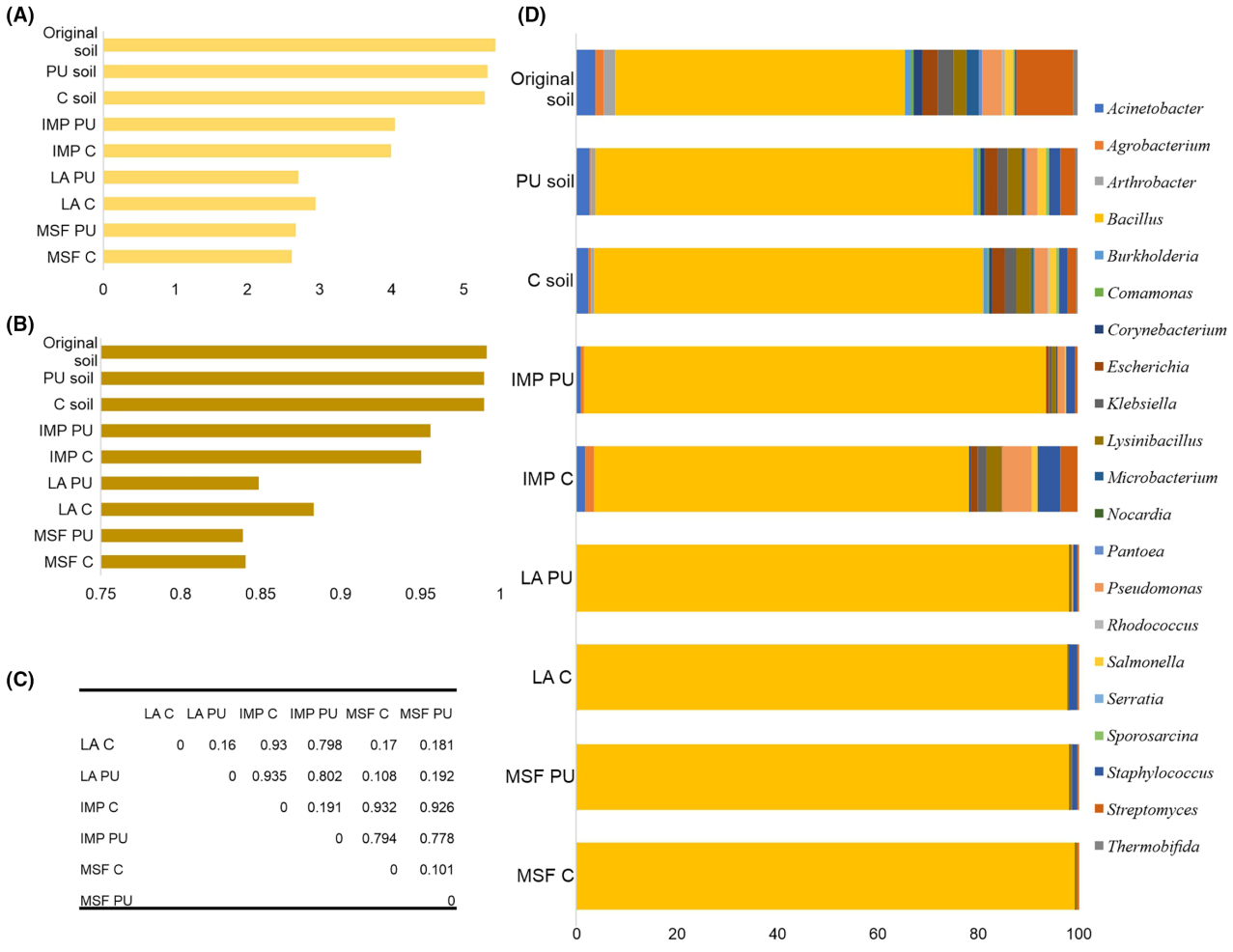

FIGURE 1 Changes in microbial diversity caused by PU enrichment of soil samples. (A) Shannon's diversity index of K, C and PU soil samples; (B) Inverse Simpson's index of K, C and PU soil samples (C) Bray–Curtis dissimilarity index of communities isolated from LA, MSF and IMP plates; (D) Distribution of PU- degrading genera within K, C and PU soil samples and IMP, LA and MSF plates.

plastic mainly polyethylene and biodegradable plastic with the latter having a more pronounced effect on the microbial community (MacLean et al., 2021; Rüthi et al., 2020), as well as significant changes in microbial community composition when exposed to PU waste (Park et al., 2023). However, all these studies focused on the composition of the 'plastisphere', the immediate proximity of plastic particles and biofilm formed as opposed to changes in bulk soil as was investigated here.

Different isolation media were investigated to determine the optimal media for obtaining the overall microbial diversity. 32,371 and 33,957 classified reads were obtained from LA plates of C (LA C) and PU soil (LA PU) respectfully, 28,019 and 23,658 from MSF plates (MSF C and MSF PU) while 37,876 and 41,929 reads were obtained from IMP plates (IMP C and IMP PU). For LA and MSF plates, the dominance of *Bacillus* strains was further exaggerated with >97% of the reads assigned to *Bacillus* species. IMP plates fared better both regarding *Bacillus* domination (although still very high) and total diversity, as the Shannon alpha diversity was 4.02 compared to an average of 2.73 for LA and MSF plates (Figure 1A). Bray–Curtis dissimilarity index was used to determine the changes between microbial communities isolated using different plates. Communities from LA and MSF plates showed high similarity with a Bray–Curtis dissimilarity index of 0.16–0.18 but communities from IMP plates had stark differences compared to LA and MSF of up to 0.93 Bray–Curtis dissimilarity index (Figure 1C). Moreover, the direct isolation on IMP-containing plates in addition to offering direct screening for potential PU degrading strains offered access to a greater diversity of microorganisms and better reflected the actual diversity of soil samples.

A collection of 62 morphologically distinct microorganisms was made by isolating microorganisms able to grow on LA and MSF media. After screening on IMP plates, only five strains showed Impranil clearing activity. On the other hand, the direct approach of isolating and screening microorganisms in one step using IMP plates yielded 59 (28 from PU soil and 31 from C soil) morphologically distinct microorganisms able to produce clearing halos (Figure S2A,B). Additionally, we evaluated the count of Impranil clearing colonies in both C and PU soils, reinforcing the finding that PU enrichment had no substantial impact on the number of potential PU degraders (as determined by Student's *t*-test).

Four strains with the most pronounced halos around growing colonies (~5 mm) were identified via 16S rDNA sequencing as *Streptomyces* species (Table S1). These strains were used in liquid culture experiments with Impranil as the sole carbon and energy source to more reliably quantify Impranil degradation and utilization. *Streptomyces* typically represent about 10%–14% of cultivable soil microorganisms (Olanrewaju & Babalola, 2019; Oskay, 2009). This genus made up less than 1% of all detected reads in C and PU soil samples, and their number decreased upon 18 months' incubation, in comparison to the original soil (Figure 1D). Nevertheless, based on the phenotypic screening results, they proved to be interesting candidates for PU degradation. *Streptomyces* strains have been previously reported to degrade Impranil in plate assays (Rüthi et al., 2023), but no in-depth study on *Streptomyces* PU degradation was performed. Recent studies have reported fungal strains capable of reaching high levels of the same Impranil degradation, such as *Embarria clematidis* that could degrade 88.8% of 1% (v/v) Impranil after 2 weeks of incubation (Khruengsai et al., 2022) while *Cladosporium* strains could degrade 80% and 94.4% in 3 days (Liu, Ahmad, et al., 2023; Zhang et al., 2022). Liquid culture Impranil degradation experiments identified *Streptomyces* strain PU10 as the most active out of the four *Streptomyces* strains tested (Figure S3). *Streptomyces* sp. PU10 was able to match and surpass the reported degradation rates with >96% Impranil degradation of 1% (v/v) Impranil after 48 h and was thus chosen for further experiments (Figure 2). *Streptomyces* sp. PU10 was able to degrade Impranil in liquid culture at range of temperatures from 25°C to 42°C with the highest initial activity at 37°C reaching at all three initial concentrations tested 0.5%, 1% and 1.5% (v/v) (Figure 2A–C).

Impranil degradation occurred rapidly with the bulk degradation taking place in the first 48 h across all tested temperatures and concentrations. Based on FTIR analysis of culture supernatants, the most intense changes occurred at wavenumber 1730 cm^{-1} belonging to carbonyl groups (C=O) in ester bonds (Zhang et al., 2022) indicating extensive ester bond cleaving (Figure 2D). Additionally, the changes at 1250, 1530 cm^{-1} and around 3400 cm^{-1} corresponding to different parts of the urethane bond (Park et al., 2023) point to changes in urethane bonds in the polymer, however, to a much lesser extent than ester bonds (Figure 2D). The release of soluble degradation products with 405.11, 611.14 and 711.19 m/z was confirmed using mass spectrometry (Figure S4), however, due to the proprietary structure of Impranil the identification of these products in beyond the scope of this research. In addition to Impranil clearing activity, PU10 was able to efficiently utilize this substrate for biomass accumulation reaching 0.61 ± 0.18 g/L cell dry weight after 3 days of incubation, compared to 0.98 ± 0.21 g/L with 1% glucose as carbon source.

Streptomyces sp. PU10 genome sequencing

Streptomyces sp. PU10 was sequenced using Oxford Nanopore MinION yielding 246,300,000 bases deposited to NCBI under accession number ASM3246384v1.

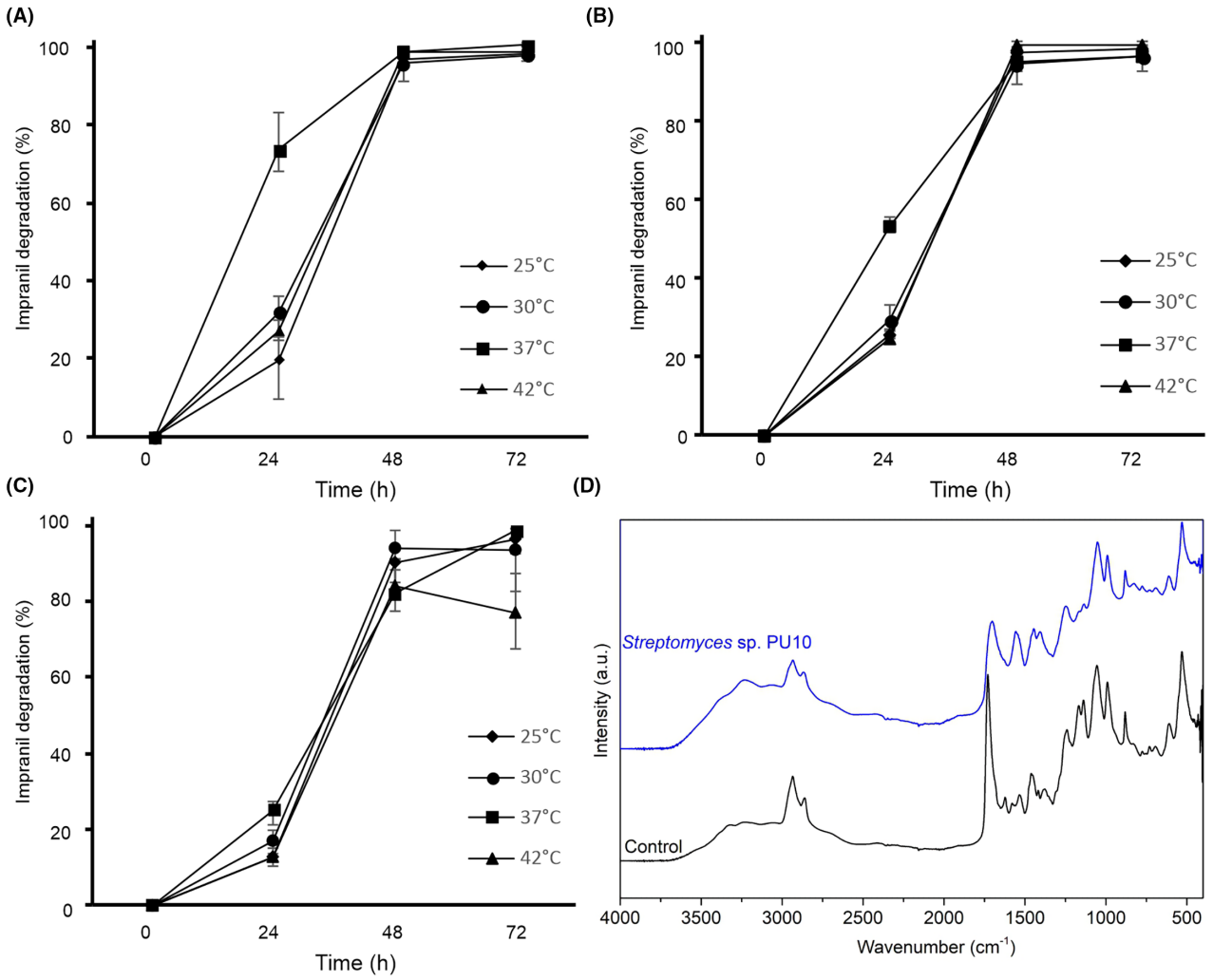

FIGURE 2 Impranil degradation by *Streptomyces* sp. PU10 in liquid culture (performed in triplicate) (A) 0.5% (v/v) of Impranil, (B) 1% (v/v) of Impranil, (C) 1.5% (v/v) of Impranil and (D) FTIR analysis of culture supernatants after 24 h of degradation.

The polished genome of *Streptomyces* sp. PU10 consisted of five contigs with a total size of 8,175,410 bp and is 96.2% complete as assessed by BUSCO using the Streptomycetales lineage. The genome consisted of 7399 predicted genes; 6781 of which were protein-coding and the closest reference genome from the Genome Taxonomy Database (GTDB) was an unclassified *Streptomyces* sp. CCM_MD2014 with ANI 95.8%. *Streptomyces* sp. PU10 genome also displayed a potential for secondary metabolite synthesis with 25 well-defined biosynthetic gene clusters detected in the genome (Table S2).

Proteins involved in Impranil degradation

The MS analysis of the extracellular fraction of *Streptomyces* sp. PU10, cultured with Impranil and glucose as the sole carbon source, resulted in the detection of 15,502 peptides overall. These peptides corresponded to an identification rate of 37.96% of the MS2 spectra, ultimately yielding 1757 reliably quantified proteins. The analysis of the intracellular fraction quantified a total of 3651 proteins, with an identification rate of 55.96% at the MS2 level. When combined the results from both fractions, a total of 3840 proteins were reliably quantified, representing 56% of the predicted proteome of *Streptomyces* sp. PU10. While slightly more proteins were detected in samples grown on glucose samples, the vast majority was present in all samples of each fraction.

Given that the degradation of polymers takes place extracellularly, a greater emphasis was given to extracellular proteins (secretome). The secretome of *Streptomyces* sp. PU10 in the presence of Impranil contained 284 overexpressed proteins including classes of proteins associated with PU degradation, 11 amidases, 9 esterases, 28 proteases and 67 oxidoreductases (Figure 3A). Interestingly, among the top 50 proteins, highest fold change oxidoreductases made up almost a quarter of the proteins suggesting that this class of enzyme may have a significant role in Impranil

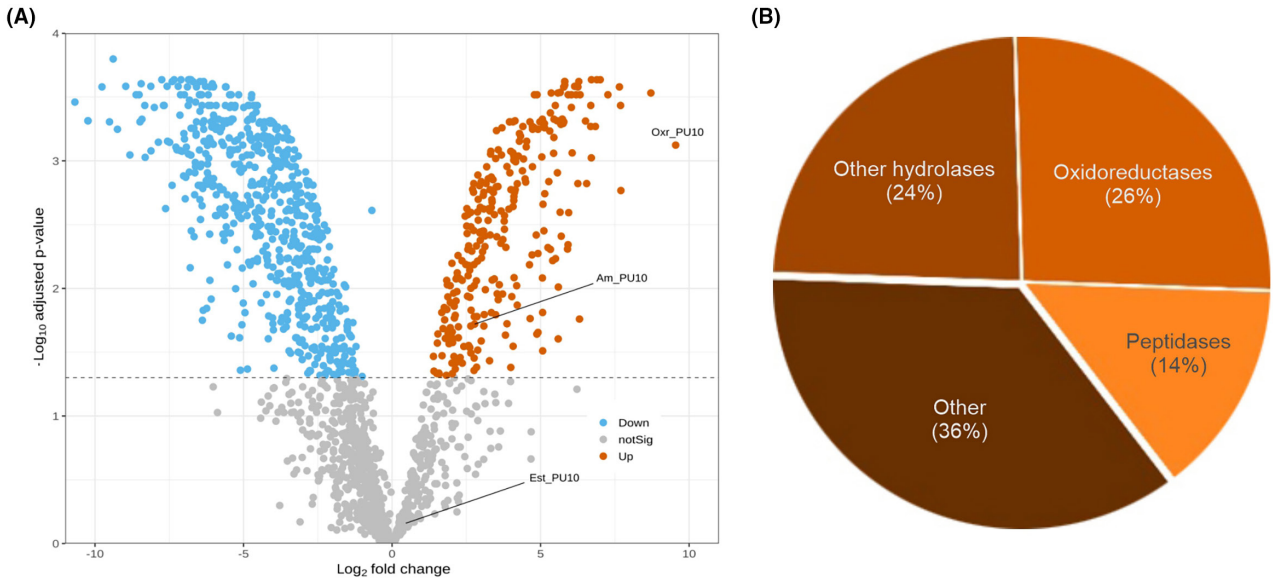

FIGURE 3 (A) Volcano plot showing the log-fold change and Benjamini–Hochberg adjusted *p*-value of each protein detected and annotated within *Streptomyces* PU10 proteome. Proteins significantly induced in Impranil or glucose are highlighted in orange and blue respectively; (B) Top 50 upregulated proteins with the highest fold change during Impranil degradation.

degradation (Figure 3B). GO enrichment analysis of overexpressed extracellular enzymes during Impranil degradation further corroborated that proteins belonging to GO terms associated with metabolic functions such as hydrolase, protease and oxidoreductase activity were significantly enriched (Table S3). Perhaps, unexpected GO terms associated with hydrolytic activity acting on glycosyl bonds were enriched as well, however, since plastic-degrading enzymes share significant similarities with non-starch plant biomass-degrading enzymes (Chen et al., 2020), it is not unlikely that enzymes involved in plastic degradation and glycoside hydrolysis are co-regulated during plastic degradation. This co-regulation could be attributed to the fact that in natural environments, these enzymes would act synergistically in the degradation of plant biomass. In contrast, the GO terms enriched in the secretome in the presence of glucose were mainly biological processes associated with biomass accumulation and protein synthesis with no enrichment in catalytic activities. Overall, GO enrichment analysis revealed that the secretome of *Streptomyces* sp. PU10 was catalytically more active during Impranil degradation and Impranil serves as an elicitor of hydrolases, proteases and oxidoreductases, as suggested by a previous report investigating the effect of Impranil on the secretome of PU-degrading *Fusarium* strains (Taxeidis et al., 2023).

To further characterize and narrow down potential PU-degrading enzymes, the secretome was compared to known PU-degrading enzymes from the PAZy database (Buchholz et al., 2022). An alpha/beta hydrolase, Est_PU10 (MDU0258016.1) with a 48 aa signal peptide for the Twin-arginine translocation pathway showed 71% identity and >80% coverage to a known PU and PET-degrading triacylglycerol lipase- Tcur_1278 from *Thermomonospora curvata* (Wei et al., 2014). This enzyme was shown to degrade commercially available thermoplastic polyester-PU at 60°C (Schmidt et al., 2017). Additionally, Est_PU10 is homologous to various PETases most closely related to metagenome-derived PETases Enzyme 607 (Erickson et al., 2022) PET40 (Zhang et al., 2023) and PHL1 and PHL2 (Sonnendecker et al., 2022) which display increased thermotolerance, a highly coveted characteristic for PETases. But Est_PU10 is also related to the benchmark enzymes LCC (Sulaiman et al., 2012), IsPETase (Yoshida et al., 2016) and PHL7 (Sonnendecker et al., 2022) as well. The relationship between Est_PU10 its most closely related PETases and the most active PETases is presented in Figure S5. The abundance of Est_PU10 was relatively low and did not significantly differ between the two assessed carbon sources indicating that Impranil did not affect the regulation of this protein under tested conditions. Although Est_PU10 has a lower abundance than other esterases detected, its high sequence identity to highly active polymer-degrading enzymes and the previously reported Impranil-degrading activity in multiple PETases (Schmidt et al., 2017) indicate that Est_PU10 is involved in Impranil degradation by cleaving the ester bonds present in the polymer, as observed in FTIR analysis (Figure 2D). The rapid and uniform degradation rates across a range of temperatures and concentrations mentioned earlier also point towards a constitutively expressed enzyme, corroborating the hypothesis that Est_PU10 is the enzyme responsible for the bulk of Impranil degradation.

On the other hand, the amidase, Am_PU10 (MDU0251973.1) from *Streptomyces* sp. PU10 was overexpressed in the presence of Impranil and showed 42% identity with >95% coverage to the metagenome-derived urethanases UMG-SP-1, UMG-SP-2 and UMG-SP-3 (Branson et al., 2023). These enzymes have been shown to efficiently degrade low-molecular weight dicarbamate molecules obtained after glycolysis of PU. During biodegradation of Impranil by *Streptomyces* sp. PU10, the bulk of the polymer is degraded via ester bond cleaving by Est_PU10, and the remaining small urethane bond-containing molecules are further degraded by Am_PU10 in a system such as the one reported by Magnin et al. employing an esterase and amidase (sequence not available) to breakdown different thermoplastic PU polymers (Magnin, Pollet, et al., 2019). Interestingly, no signal peptide was predicted for Am_PU10 and it was detected at a higher concentration in the intracellular fraction, suggesting its presence in the secretome can be attributed to cell lysis or alternate excretion mechanisms since GO terms related to transmembrane transport were enriched (Table S3). The degradation of small urethane bond-containing molecules is most likely further assisted by two more overexpressed alpha/beta hydrolases MDU0254145.1 and MDU0258220.1 homologous (37% identity with >95% coverage) to an ethyl carbamate degrading gamma-lactamase from *Microbacterium hydrocarbonoxydans* (Kang et al., 2021). Overall, these results point to soluble Impranil degradation products eliciting the expression of urethane bond degrading hydrolases.

The protein that showed the highest differential expression, with a logFC 9.54 change (748-fold increase), was an oxidase containing a FAD-binding domain, Oxr_PU10 (MDU0255931.1). Oxidoreductases are a vastly under-represented class of enzymes in plastic degradation studies of hydrolysable polymers, with most of the research conducted on the degradation of polyolefins. The proposed mechanism of oxidoreductases in plastic degradation includes hydroxylation of C-C bond yielding primary or secondary alcohols which are eventually oxidized to carboxylic acids and metabolized by microorganisms (Han et al., 2024). Recently, a laccase-mediated system employing the laccase from *Trametes versicolor* was used to efficiently degrade both PU foams and thermoplastics employing a different mechanism compared to hydrolases (Magnin et al., 2021) demonstrating oxidoreductases can be used for a much broader range of polymers. Oxr_PU10 is a distant homologue of Oxr-1 oxidoreductase from *Bacillus velezensis*, identified through proteomics. The purified, heterologously expressed, Oxr-1 enzyme was capable of degrading Impranil and polybutylene adipate terephthalate (PBAT) (Gui et al., 2023). The lack of experimental and sequencing data hampers the discovery of novel plastic-degrading oxidoreductases but based on the results presented in this work oxidoreductases may present an untapped pool of PU-degrading enzymes acting in unison with different hydrolases. Moreover, recent reports have identified decarboxylases acting together with hydrolases to breakdown commercial PU (Bhavsar et al., 2023) validating the notion that complex polymers such as PU require multiple enzymes with diverse functions for effective degradation.

Metabolic responses and secondary metabolite production upon growth on Impranil

Streptomyces strains are not only widely recognized for their role as a valuable reservoir of medically significant compounds including antibiotics, antifungals, immunosuppressants and anticancer drugs (Alam et al., 2022) but they also boast a diverse enzymatic arsenal for various biotechnological applications (Spasic et al., 2018). This combination of enzymatic and secondary metabolite-producing capabilities has been leveraged for the upcycling of paper, textile (Cuebas-Irizarry & Grunden, 2023) and agro-industrial waste (Kashiwagi et al., 2017). To understand how Impranil degradation intermediates are incorporated into the primary and secondary metabolism of *Streptomyces* sp. PU10 overexpressed proteins detected in the intracellular fraction were assigned to KEGG metabolic pathways using KOBAS and BlastKoala. KOBAS enrichment analysis did not identify any significant changes in metabolic pathway activity (after multiple test correction) compared to culturing with glucose as the sole carbon source. This finding coupled with aforementioned comparable biomass yields signifies that *Streptomyces* sp. PU10 can efficiently utilize Impranil with only subtle metabolic changes. However, based on the distribution of overexpressed proteins the most likely path to incorporating soluble alcohols, the main degradation intermediates of Impranil (Biffinger et al., 2015) and other polyester-PU (Utomo et al., 2020) is via the fatty acid degradation pathway since fatty acid degradation and metabolism pathways had enrichment ratios of 0.33 and 0.29 respectively (data not shown). This pathway has been also suggested in the case of the fungal strain *Cladosporium halotolerans* 6UPA1 (Zhang et al., 2022). Alcohols are oxidized to fatty acids by a myriad of oxidoreductases detected including alcohol and aldehyde oxidases and directed to the fatty acid degradation pathway (Figure 4). Nine overexpressed proteins were assigned to fatty acid metabolism, crucially, FadD (MDU0257621.1) the acyl-CoA synthetase responsible for initiating the pathway by activating fatty acids was overexpressed. FadD is known to have broad substrate specificity both in *Streptomyces* and other microorganisms acting on both aliphatic and aromatic long-chain substrates (Hume et al., 2009) and has been linked to effective secondary metabolite production (Banchio & Gramajo, 2002).

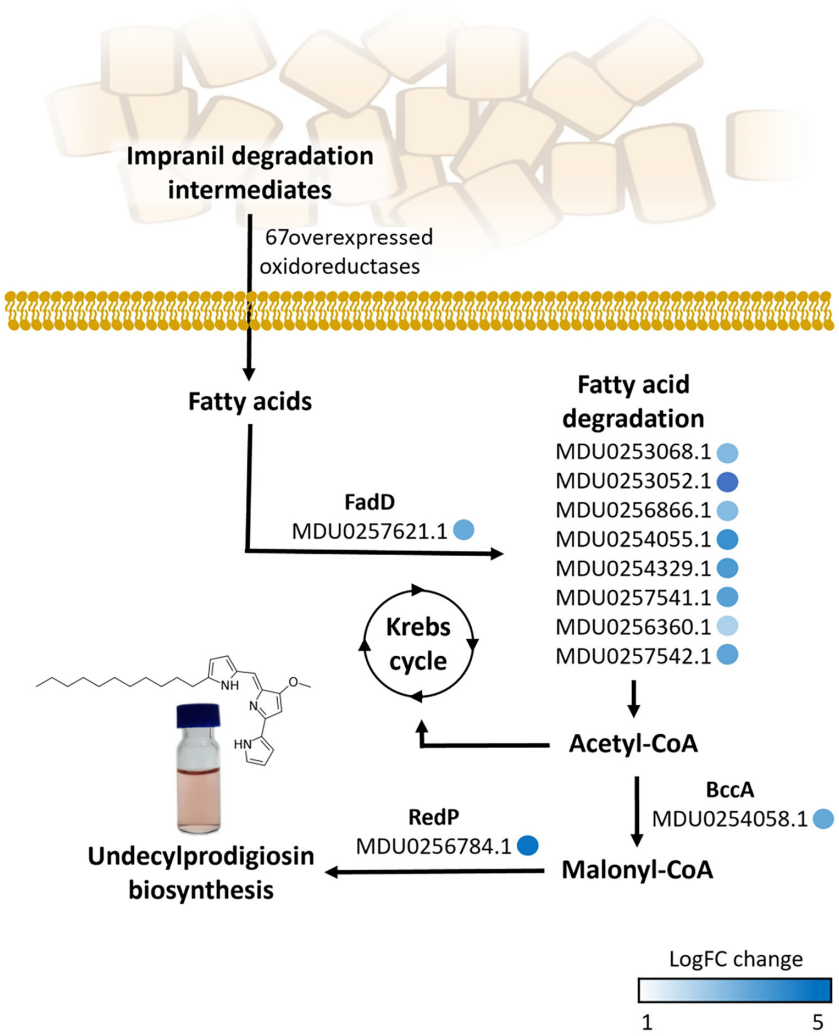

FIGURE 4 Proposed metabolic pathways and proteins involved in Impranil degradation, intermediate metabolism and secondary metabolite production by *Streptomyces* sp. PU10

In addition to MDU0257621.1, two more copies of FadD (MDU0253472.1 and MDU0253870.1) have been detected during growth on Impranil. The resulting acetyl-CoA form fatty acid degradation is then directed to the Krebs cycle but is also used as a substrate for the upregulated acetyl-CoA carboxylase BccA (MDU0254058.1) and transformed to malonyl-CoA. Malonyl-CoA and BccA are essential for bridging the gap from primary to secondary metabolism (Laakel et al., 1994) and have been identified as the link between fatty acid metabolism and polyketide biosynthesis (Summers et al., 1995). Metabolic engineering strategies overexpressing acetyl-CoA carboxylase and increasing the supply of malonyl-CoA have been successfully utilized for increased antibiotic production (Liao et al., 2022; Zabala et al., 2016). All the processes mentioned above point to Impranil degradation intermediates being incorporated into the central metabolism of *Streptomyces* sp. PU10 and funnelled to polyketide production. Five polyketide clusters were identified in the genome of *Streptomyces* sp. PU10 comprised of two polyketide type III synthase-containing clusters for germicidin and flaviolin/1,3,6,8-tetrahydroxynaphthalene, two polyketide type II synthase-containing clusters producing unknown compounds and the well-studied undecylprodigiosin (UP) cluster (Table S2). In the genome of *Streptomyces* sp. PU10, all 22 genes responsible for the biosynthesis of UP and its characteristic tri-pyrrole structure were identified. UP has anticancer, antimicrobial, antioxidative and UV-protective properties (Ho et al., 2007; Stankovic et al., 2012). Ten proteins involved in the UP biosynthesis pathway were detected through proteomic analysis while RedP (MDU0256784.1) the enzyme initializing UP synthesis by catalysing the condensation of a malonyl group (Hu et al., 2016) was upregulated (28-fold increase). Plastics triggering UP production was observed in *S. coelicolor* exposed to PLA microplastics as well but no investigation on the mechanism of this response was conducted (Liu, Fang, et al., 2023). The production of UP by *Streptomyces* sp. PU10 was confirmed by UV/VIS and LC–MS analysis after prolonged incubation (7 days) (Figure S6A,B). It is important to note a relatively low amount (2 ± 0.2 mg/L) of UP was produced by *Streptomyces* sp. PU10, possibly due to aberrant RedJ expression or tight control of this biosynthetic cluster (Whicher et al., 2011).

A comparable amount was obtained from the MSF medium, while no UP was detected when *Streptomyces* sp. PU10 was grown in MSM containing glucose as sole carbon source. Overall, using Impranil degradation intermediates as a carbon source led to the accumulation of malonyl-CoA which is an important precursor of polyketide biosynthesis signifying PU hydrolysates both from chemical and biological depolymerization could be used for polyketide production. Similarly, feeding soybean oil as a supplementary carbon source to *S. tsukubaensis* led to an increase in FK506 polyketide production by increasing FK506 precursor supply (Wang et al., 2017).

Regarding other non-polyketide secondary metabolites, among the upregulated proteins were proteins involved in terpenoid biosynthesis as well. MDU0254329.1, MDU0257541.1 and MDU0255453.1 are involved in terpenoid backbone biosynthesis suggesting terpenoid compounds abaflavenone, hopene and isorenieratene, identified by antiSMASH are being produced (Table S2). The overexpressed carotenoid phi-ring synthase-crtU (MDU0257749.1) catalyses the conversion of β-isorenieratene to isorenieratene thus confirming this diaromatic carotenoid, which can be used to protect from UV-induced DNA damage (Wagener et al., 2012), is likely being produced. Interestingly, a recently identified cluster for ashimide A and ashimide B poorly known non-ribosomal peptides with cytotoxic activity (Shi et al., 2019) was overexpressed in 10 out of 22 proteins directly involved in ashimide biosynthesis upregulated. The triggers for ashimide production are still unknown and ashimides have only been detected through heterologous expression in alternative hosts pointing to the possibility of using PU degradation intermediates for producing novel compounds of interest from *Streptomyces* orphan biosynthetic gene clusters.

CONCLUSIONS

In conclusion, our investigation delved into the effect of PU waste on the diversity of microbial communities and identified a promising candidate for PU degradation and upcycling. *Streptomyces* sp. PU10 degraded Impranil by a combination of at least three enzymes and incorporated the degradation products into its primary and secondary metabolism. Noteworthy was the production of undecylprodigiosin and potentially other valuable natural products signalling a promising opportunity for PU upcycling to highly valuable bioactive compounds by employing *Streptomyces* strains. This comprehensive study not only enhances our understanding of PU waste impact on microbial ecosystems but also provides valuable insights for developing sustainable solutions for the utilization of PU waste.

AUTHOR CONTRIBUTIONS

Brana Pantelic: Investigation; methodology; validation; writing – original draft; writing – review and editing. **Romanos Siaperas:** Investigation; methodology; writing – original draft. **Clémence Budin:** Investigation; methodology; writing – original draft. **Tjalf de Boer:** Investigation; methodology; writing – original draft. **Evangelos Topakas:** Investigation; methodology; resources; supervision; writing – review and editing. **Jasmina Nikodinovic-Runic:** Conceptualization; funding acquisition; methodology; resources; supervision; validation; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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