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IZOLACIJA BAKTERIJA I KARAKTERIZACIJA NJIHOVIH ENZIMA SA CILJEM RAZVIJANJA BIOKATALIZATORA ZA BIODEGRADACIJU PLASTIKE

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ISOLATION OF BACTERIA AND CHARACTERIZATION OF THEIR ENZYMES TO DEVELOP BIOCATALYSTS FOR PLASTIC BIODEGRADATION

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Izolacija bakterija i karakterizacija njihovih enzima sa ciljem razvijanja biokatalizatora za biodegradaciju plastike

SAŽETAK

Usled masovne proizvodnje i neadekvatnog korišćenja plastike došlo je do rastućeg globalnog problema akumulacije plastičnog otpada. Različite strategije su primenjene kako bi se ovaj problem rešio, ali upotreba mikroorganizama i njihovih enzima za razgradnju plastičnih polimera bio je fokus istraživača u proteklih nekoliko decenija. Glavni ciljevi ove doktorske disertacije bili su izolacija i karakterizacija bakterijskih sojeva koji razgrađuju plastiku, prevashodno poli(etilentereftalat) (PET) i poli(uretane) (PU), njihov dalji razvoj u efikasne biokatalizatore, identifikacija enzima odgovornih za navedenu aktivnost i istraživanje potencijalnih metoda biološke valorizacije plastičnog otpada.

Ispitivanjem laboratorijske kolekcije mikroorganizama tri bakterijska soja su odabrana kao potencijalni biokatalizatori za razgradnju plastike. *Bacillus subtilis* BPM12 efikasno je formirao biofilm na PET plastici i pokazao izuzetnu aktivnost na bis(2-hidroksietil) tereftalatu i različitim supstratima, strukturno sličnim PET oligomerima i monomerima. Kroz heterolognu i homolognu ekspresiju, potvrđeno je da je enzim Bpm12CE ključan za ovu aktivnost. Delovanjem više različitih enzima, *Amycolatopsis mediterranei* ISP5501 razgrađivao je različite vrste PU, a upotrebom model jedinjenja PU-7 potvrđena je i hidroliza uretanske veze. *Streptomyces* sp. PU10 pokazao se kao jedan od najaktivnijih, do sada prijavljenih, sojeva za hidrolizu PU disperzije – Impranil sa preko 96% razgradnje za 72 h. Na osnovu rezultata kvantitativne proteomike zaključeno je da su u razgradnju PU uključene esteraza, amidaza i oksidaza ovog soja, dok se produkti razgradnje inkorporiraju u primarni metabolizam *Streptomyces* sp. PU10 i usmeravaju ka biosintezi poliketida. Konačno, uspostavljena je i strategija za konvertovanje biorazgradivih skrob/poli(vinil-alkohol) filmova u jedinjenja sa dodatom vrednošću: biopolimer poli(hidroksibutirat) i biološki aktivni biopigment undecilprodigiozin.

Ključne reči: Plastika, PET, PU, Biodegradacija, Biokataliza, *Bacillus*, *Amycolatopsis*, *Streptomyces*, Undecilprodigiozin

Naučna oblast: Biologija
Uža naučna oblast: Molekularna biologija prokariota

Isolation of bacteria and characterization of their enzymes to develop biocatalysts for plastic biodegradation

ABSTRACT

Due to mass production and inadequate plastic usage, a growing issue of global plastic waste accumulation has emerged. Various strategies have been employed to address this problem, but using microorganisms and their enzymes for plastic polymer degradation has captivated researchers' attention in recent decades. The primary goal of this doctoral dissertation was the isolation and characterization of bacterial strains capable of degrading plastics, mainly poly(ethylene terephthalate) (PET) and poly(urethanes) (PU), and their further development into efficient biocatalysts, the identification of enzymes responsible for this activity and exploration of potential methods for the biological valorization of plastic waste.

From a laboratory collection of microorganisms, three bacterial strains were selected as potential biocatalysts for plastic degradation. *Bacillus subtilis* BPM12 efficiently formed biofilm on PET plastic, exhibiting exceptional activity on bis(2-hydroxyethyl) terephthalate and structurally similar PET oligomers and monomers. Through heterologous and homologous expression, the enzyme Bpm12CE was confirmed to be crucial for this activity. *Amycolatopsis mediterranei* ISP5501 degraded various types of PU using multiple enzymes, and the hydrolysis of the urethane bond was confirmed using the model compound PU-7. *Streptomyces* sp. PU10 demonstrated high activity, with over 96% degradation of PU dispersion - Impranil within 72 hours. Quantitative proteomics results indicated that an esterase, amidase, and oxidase of this strain were involved in PU degradation, with the degradation products being incorporated into the primary metabolism of *Streptomyces* sp. PU10 and directed towards polyketide biosynthesis. Finally, a strategy was established for converting biodegradable starch/poly(vinyl alcohol) films into value-added compounds: the biopolymer poly(hydroxybutyrate) and the biologically active biopigment undecylprodigiosin.

Keywords: Plastic, PET, PU, Biodegradation, Biocatalysis, Esterase, *Bacillus*, *Amycolatopsis*, *Streptomyces*, Undecylprodigiosin

Scientific filed: Biology
Scientific subfield: Molecular biology of prokaryotes

NAJČEŠĆE KORIŠĆENE SKRAĆENICE

PE- poli(etilen)

PP- poli(propilen)

PVC- poli(vinil-hlorid)

PET- poli(etilentereftalat)

PU- poli(uretan)

PS- poli(stiren)

PAH- poliaromatični ugljovodonici

PM- eng. *particulate matter*

PHA- poli(hidroksialkanoat)

PCL- poli(kaprolakton)

PBS- poli(butilen-sukcinat)

PLA- poli(mlečna kiselina)

PVA- poli(vinil-alkohol)

PHB- poli(hidroksibutirat)

PHO- poli(hidroksioktanoat)

IUPAC- Međunarodna unija za čistu i primenjenu hemiju (eng. *International Union of Pure and Applied Chemistry*)

ISO- Mađunarodna organizacija za standardizaciju (eng. *International Standardization Organization*)

IsPETaza- PETaza iz *Ideonella sakaiensis*

TPA- tereftalna kiselina

MHET- (2-hidroksietil)-tereftalat

BHET- bis(2-hidroksietil)-tereftalat

LCC- eng. *Leaf Compost Cutinase*

PAZy - Baza podataka enzima aktivnih na plastici (eng. *The Plastics-Active Enzymes Database*)

# SADRŽAJ

1. UVOD

1.1. Plastika i plastični otpad

Plastika je oblikovala moderno društvo i postala nezamenljiv materijal. Izdržljivost, hemijska inertnost, hidrofobnost, niska specifična težina, lakoća manipulisanja i pre svega niska cena ovih sintetičkih polimera omogućila je upotrebu plastike u najrazličitijim industrijama (Wei and Zimmermann, 2017). Većina plastike (>98%) se dobija preradom neobnovljivih fosilnih resursa i zajedno sa energijom koju je potrebno utrošiti u proizvodnji čini skoro 8% globalne potrošnje fosilnih goriva. Pored gasova staklene, bašte proizvodnja plastike podrazumeva i ispuštanje ugljen-monoksida, azotovih oksida i cijanovodonične kiseline u atmosferu (Ali et al., 2021). Najčešće korišćene vrste plastike su poli(etilen) (PE) (26,9%), poli(propilen) (PP) (19,3%), poli(vinil-hlorid) (PVC) (12,9%), poli(etilentereftalat) (PET) (6,2%), poli(uretani) (PU) (5,5%) i poli(stiren) (PS) (5,3%) (**Slika 1A**) koji zajedno čine skoro ¾ plastičnih polimera proizvedenih svake godine (PlasticsEurope, 2022) (**Slika 1B**). Od toga se najveći deo, 44%, koristi u svrhe pakovanja, 18% u građevinskoj industriji, 8% u automobilskoj industriji, 7% u elektronici, 7% u svakodnevnim predmetima i 4% u poljoprivredi i baštovanstvu (PlasticsEurope, 2022). Od početka dvadesetog veka kada je sintetisan prvi plastični polimer, i 50-ih godina kada je otpočela masovna proizvodnja plastike pa do 2015. godine proizvedeno je oko 8300 Mt plastike (Geyer et al., 2017). Na godišnjem nivou se proizvodi između 380 i 450 Mt plastike, međutim, predviđeno je da će se proizvodnja udvostručiti u sledećih nekoliko decenija (Stegmann et al., 2022, Bergmann et al., 2022).

Sa masovnom proizvodnjom otpočela je i akumulacija plastičnog otpada tako da je od sredine 50-ih godina do sada proizvedeno 5800 Mt plastičnog otpada na svetskom nivou. Najveći deo ovog otpada završio je u prirodnom okruženju i deponijama (4900 Mt), manji deo bio je spaljen (800 Mt) i samo 600 Mt ili 9% sve plastike ikada proizvedene je reciklirano. Od toga samo 10% reciklirane plastike je bilo reciklirano više od jedanput (Geyer et al., 2017). Plastični otpad je postao toliko sveprisutan u okruženju da su neki istraživači predložili prisustvo plastike kao geološki indikator Antropocena, epohe u kojoj živimo (Zalasiewicz et al., 2016). Akumulacija plastičnog otpada u kopnenim i vodenim ekosistemima dovodi do dobro dokumentovanih negativnih efekata na živi svet. Ovi efekti se prevashodno ogledaju u ispuštanju toksičnih monomera i aditiva korišćenih u proizvodnji plastike. Jedinjenja kao što su bisfenol A[1] i bis(2-etiheksil) benzen-1,2-dikarboksilat su identifikovana kao endokrini disruptori koji utiču na reproduktivni sistem, a mogu zagaditi podzemne vode kroz neadekvatno obezbeđene deponije (North and Halden, 2013). Diizocijanati i diamini koji se ispuštaju iz PU pena takođe predstavljaju kancerogene i supstance visokog rizika (Luft et al., 2017). Drugi način na koji plastični otpad može da ispolji negativne efekte jeste prosto upetljavanje različitih akvatičnih organizama u ovaj otpad, najčešće kornjača, vodenih sisara i ptica (Tanaka et al., 2013). Slučajnim konzumiranjem plastike dolazi do blokade digestivnog trakta i smanjene mobilnosti kod životinja. Do danas je prijavljeno

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[1] IUPAC- 4,4'-(propan-2,2-dil)difenol

više od 690 morskih vrsti životinja u čijem digestivnom traktu su pronađeni tragovi plastike (Provencher et al., 2017).

Mehaničkim oštećenjima i delovanjem abiotičkih faktora kao što su temperatura i UV zračenje dolazi do formiranja čestica mikro- i nanoplastike sa još nedovoljno istraženim posledicama po životnu sredinu i ljudsko zdravlje (Anik et al., 2021). Ono što međutim jeste poznato je da ove mikroskopske čestice mogu služiti kao vektori za prenos različitih zagađujućih supstanci raznolikog porekla, čak i patogenih mikroorganizama. Hidrofobna površina mikroplastike omogućava adsorpciju jedinjenja kao sto su poliaromatični ugljovodonici (PAH), polihlorovani bifenili, toksični metali i drugih organskih jedinjenja (Liu et al., 2016, Caruso, 2019).
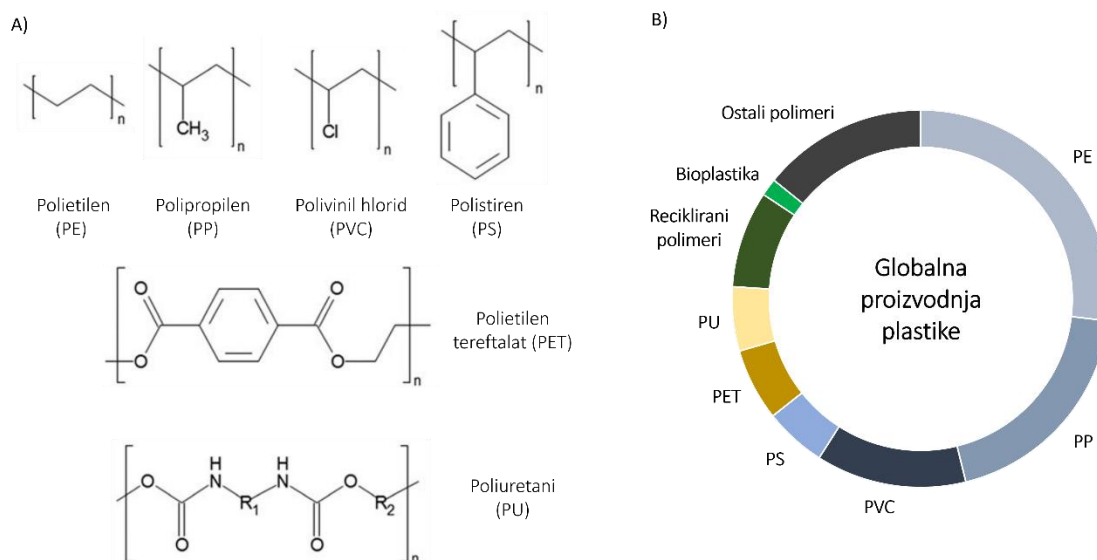
Slika 1. A) Hemijske strukture najzastupljenijih plastičnih polimera; B) Udeo različitih plastičnih polimera u godišnjoj proizvodnji na svetskom nivou.

Imajući u vidu kontinualnu akumulaciju plastičnog otpada i štetne posledice po životnu sredinu i ljudsko zdravlje razvijeni su različiti pristupi u rešavanju ovog kompleksnog problema, uključujući strategije za mehaničko i hemijsko recikliranje, biotehnološki tretman plastičnog otpada, kao i napori da se plastika zameni održivim materijalima.

1.2. Strategije smanjivanja akumulacije plastičnog otpada

Smanjenje upotrebe, višekratno korišćenje, recikliranje i povrat sirovina (eng. *Reduce, Reuse, Recycle and Recover*) predstavljaju osnovne koncepte u strategijama smanjenja bilo kog otpada. Prva dva koncepta odnose se na način upotrebe određenih predmeta i proizvoda sa ciljem da ne dođe do stvaranja otpada na prvom mestu. Recikliranje i povrat sirovina predstavljaju načine zbrinjavanja već nastalog otpada (Yu et al., 2021). Imajući u vidu neefikasnost strategija usmerenih ka smanjenju stvaranja novog plastičnog otpada najviše pažnje biće posvećeno načinima recikliranja i valorizacije plastike. Najjednostavnija i najšire primenjivana strategija za

smanjivanje količine plastičnog otpada jeste spaljivanje. Kao što je već navedeno, 12% od sve plastike ikada proizvedene je spaljeno u svrhe dobijanja energije (Geyer et al., 2017). Iako ovaj pristup predstavlja jednu od ekonomski najefikasnijih strategija, ekološka održivost i „zelenost" ovog procesa je vrlo problematična. Tokom sagorevanja plastika ispuštaju se PM čestice (eng. *particulate matter*), toksična jedinjenja kao što su toksični metali, aldehidi, ugljen-monoksid, azotovi oksidi, furan[2] i PAH zajedno sa gasovima staklene bašte ugljen-dioksidom i metanom (Ali et al., 2021). Usled navedenog, spaljivanje plastike se postepeno zamenjuje održivijim metodama kao što su mehaničko, hemijsko i biološko recikliranje, tj. biotehnološki tretman.

Mehaničko recikliranje plastike je proces koji uključuje fizičko usitnjavanje i obradu plastičnih materijala, koje se može i ne mora izvoditi na povišenim temperaturama, kako bi se oni ponovno koristili. Plastika se melje na sitnije komade, koje se nazivaju granulama ili čipsom, a koje mogu biti iskorišćene za proizvodnju novih plastičnih proizvoda ili kao dodaci u građevinskoj industriji, prevashodno za asfalt i beton (Ragaert et al., 2017). Međutim, nakon mehaničkog recikliranja dobijeni polimeri su lošijih karakteristika od originalnih polimera što umnogome ograničava ovaj tip recikliranja. Ovaj gubitak poželjnih karakteristika nakon mehaničkog recikliranja često se naziva i „downcycling" (Jehanno et al., 2022, Vogt et al., 2021). Stoga se strategije koje za rezultat imaju dobijanje nepromenjenih polimera ili jedinjenja sa dodatom vrednošću smatraju znatno primamljivijim.

Hemijsko recikliranje obuhvata različite tehnologije koje razgrađuju polimere plastike na molekulskom nivou, omogućavajući ponovnu upotrebu monomera i/ili smeše monomera za proizvodnju novih plastika (istih karakteristika kao i originalni polimer) ili čak jedinjenja sa dodatom vrednošću. Piroliza, hidroliza, metanoliza, i glikoliza su neke od najčešće korišćenih strategija (Thiounn and Smith, 2020). Piroliza podrazumeva termalnu degradaciju plastičnih polimera u odsustvu kiseonika nakon čega se dobijaju voskovi i gasovi koje se mogu dalje prečišćavati i koristiti u različite svrhe. Ovaj tip recikliranja uspešno je razvijen za PET, PS, PE, PP i PVC (Honus et al., 2018). Hemijska hidroliza plastike predstavlja reakciju sa vodom (hidrolizu) na povišenim temperaturama sa ili bez prisustva katalizatora u neutralnim, kiselim ili baznim sredinama (Alonso et al., 1999). Nažalost, ovaj proces, iako efikasan, ograničen je samo na polimere koji u svojoj strukturi imaju veze koje mogu da se hidrolizuju. Metanoliza predstavlja reakciju plastike sa metanolom na povišenim temperaturama i pri povišenom pritisku, a za rezultat ima formiranje monomera (Genta et al., 2005). Naposletku, glikoliza predstavlja reakciju plastike sa glikolima i za rezultat ima formiranje smeše monomera, oligomera i različitih poliola (Suh et al., 2000). Ovde su pomenute samo neke od mnogobrojnih metoda za hemijsko recikliranje plastike, međutim, upotreba ovih metoda u velikom broju slučajeva nije moguća zbog niza otežavajućih faktora, pre svega ekonomskih i ekoloških. Svi ovi procesi zahtevaju povišenu temperaturu, povišen pritisak ili upotrebu toksičnih katalizatora, a prečišćavanje komplikovanih reakcionih smeša iziskuje dodatne utroške resursa i energije (Thiounn and Smith, 2020, Vogt et al., 2021). U skorije vreme razvijeni su hemijski procesi koji zahtevaju manji utrošak energije, a kako bi procesi bili ekonomski isplativi, kao krajnji proizvod imaju jedinjenja sa dodatom vrednošću, koncept nazvan „upcycling" (Morado et al., 2023, Liu et al., 2023c).

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[2] IUPAC- 1-oksaciklopenta-2,4-dien

Razgradnja plastičnih polimera na molekulskom nivou može se postići i upotrebom hemijskih katalizatora ali i upotrebom bioloških katalizatora - biokatalizom. Biokataliza predstavlja primenu enzima i mikroorganizama kao katalizatora različitih hemijskih reakcija, koristeći ih u svrhe za koje nisu evoluirali (odeljak 1.3). Kao alternativni pravac smanjenja akumulacije teško razgradivog plastičnog otpada ulažu se i napori da se plastika zameni održivim materijalima poput biorazgradive bioplastike (odeljak 1.4).

1.3. Biotehnološki tretman plastičnog otpada

Oblast biokatalize je dostigla svoj trenutni nivo razvoja i upotrebu na industrijskoj skali kroz nekoliko talasa istraživanja i inovacija. Prvi talas, koji je počeo pre više od jednog veka, uključivao je jednostavno korišćenje ekstrakata biljaka ili mikroorganizama koji sadrže enzimsku aktivnost od interesa za određenu reakciju. Drugi talas, sa početka 80-ih godina prošlog veka, omogućen je genetičkim inženjerstvom, kloniranjem i ekspresijom enzima od interesa u odgovarajućim mikroorganizmima kao domaćinima. Od izuzetne važnosti je bio i početak primene usmerene mutageneze kako bi se izmenila sekvenca gena sa ciljem poboljšanja svojstava enzima. Treći talas počeo je sa razvijanjem naprednih metoda proteinskog inženjerstva tokom 90-ih, poput lančane reakcije polimeraze sklone greškama (eng. *error prone PCR*) u kombinaciji sa metodama visoke propusnosti za testiranje različitih varijanti enzima, koje se sada često označavaju kao dirigovana evolucija. Uspesi navedenih pristupa omogućili su da se biokataliza danas koristi za različite primene u industriji hrane, poljoprivredi, organskoj sintezi finih hemikalija i veštačkih ukusa, a posebno u proizvodnji farmaceutski značajnih jedinjenja (Bornscheuer, 2018, Bornscheuer et al., 2012). Uzevši u obzir uspehe i prilagodljivost, biokataliza se istakla kao potencijalna strategija za tretman plastičnog otpada.

Pretpostavljeni mehanizam razgradnje plastike u spoljašnjoj sredini podrazumeva sinergističko dejstvo abiotičkih i biotičkih faktora. Mehanički stres, toplota i UV zračenje dovode od fragmentacije polimera i promena funkcionalnih grupa na površini i čime postaju dostupniji za dejstvo vanćelijskih enzima - depolimeraza. Dejstvom depolimeraza dugački polimerni lanci se hidrolizuju do monomera koji dalje bivaju asimilovani od strane mikroorganizama i finalno bivaju mineralizovani do ugljen-dioksida i vode (Ali et al., 2021) (**Slika 2**). Ovaj proces, prema nekim procenama, može da traje i preko 500 godina upravo zbog karakteristika koje su učinile plastiku toliko rasprostranjenom i nezamenljivom u svakodnevnom životu (Chamas et al., 2020). Visok nivo hidrofobnosti i visoka molekulska masa onemogućavaju efikasnu asimilaciju od strane mikroorganizama i adsorpciju enzima na površinu polimera (Arutchelvi et al., 2008, Wei and Zimmermann, 2017). Visoka kristaliničnost (uređenost lanaca polimera), hemijska inertnost i glatka površina polimera plastike onemogućavaju formiranje biofilma i dodatno sputavaju efikasnu (bio)degradaciju plastičnih polimera (Cregut et al., 2013). Uprkos brojnim preprekama i nedostacima ovi procesi poslužili su kao inspiracija za razvijanje bioloških/biotehnoloških rešenja za smanjenje plastičnog otpada. Uzevši u obzir mane mehaničkih i hemijskih metoda (odeljak 1.2), biotehnološka rešenja obećavaju kao ekološki održiva i ekonomski isplativa strategija za rešavanje problema plastičnog otpada. Niske temperature, ambijentalni pritisak, energetska efikasnost i

relativno lako prečišćavanje proizvoda reakcije navode se kao ključne prednosti korišćenja mikroorganizama i njihovih enzima u ove svrhe (Qi et al., 2021).

Prve indikacije da mikroorganizmi mogu da razgrađuju plastiku uočene su oko 30 godina nakon početka masovne proizvodnje plastike (Fields et al., 1974). Od tog trenutka do 2010. godine identifikovano je 219 različitih mikroorganizama sa sposobnošću da u nekoj meri razgrade plastiku, a ovaj broj se skoro udvostručio na 436 vrsta sredinom 2020. godine. Ovakav rastući trend se nastavlja i danas (Rong et al., 2024, Ou et al., 2024, Bhanot et al., 2024). Najveći deo opisanih mikroorganizama su bakterije sa oko 65%, a manji deo čine gljive i kvasci sa oko 35%. Zanimljivo je napomenuti da se sve do sada identifikovane bakterije sposobne da razgrađuju plastiku nalaze u samo pet razdela. Proteobacteria, Actinobacteria i Firmicutes čine većinu, dok je znato manje predstavnika identifikovano u razdelima Bacteroidetes i Cyanobacteria. Sa druge strane, gljive sposobne da razgrađuju plastiku klasifikovane su kao pripadnici Ascomycota, Basidiomycota i Mucoromycota (Gambarini et al., 2021). Mikroorganizmi koji razgrađuju plastiku su prisutni na svim geografskim širinama i najrazličitijim staništima s obzirom da su pronađeni u zemljištu (Shah et al., 2016), kompostu (Hu et al., 2010), deponijama i smetlištima (Yoshida et al., 2016), postrojenjima za preradu otpadnih voda (Skariyachan et al., 2018), sedimentima iz tropskih i muljevitih močvara (Auta et al., 2018), glečerima (Urbanek et al., 2018), okeanima (Gui et al., 2023), crevima insekata (Kim et al., 2019a, Yang et al., 2023), i drugim staništima. Kao najproduktivnije stanište u pogledu mikroorganizama koji mogu da razgrađuju plastiku identifikovano je zemljište sa 27,8% mikroorganizama prijavljenih da razgrađuju plastiku, za kojim slede deponije (9,6%) i kompost (5,3%) (Gambarini et al., 2021).
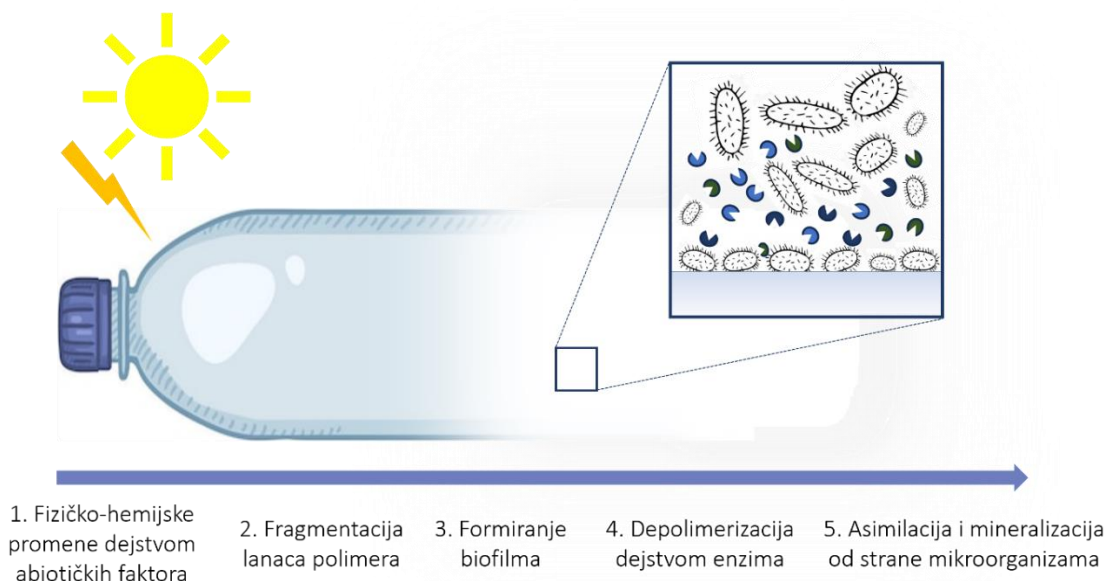
Slika 2. Shematski prikaz procesa razgradnje plastike u životnoj sredini.

Mikroorganizmi vrše razgradnju plastike dejstvom različitih, pre svega, vanćelijskih enzima. Esteraze (EC 3.1), kutinaza (EC 3.1.1.74), lipaze (EC 3.1.13), lakaze (EC 1.10.3.2) i peroksidaze

(EC 3.11.1) su najčešći tipovi enzima asociranih sa razgradnjom plastike (Nikolaivits et al., 2021) (**Tabela 1**). Imajući u vidu sličnosti između sintetičkih i prirodnih polimera koji čine prirodnu, predominantno biljnu biomasu (npr. celuloza, lignin, kutin, hitin) i klase enzima koje učestvuju u razgradnji plastike, smatra se da enzimi koji razgrađuju plastiku predstavljaju promiskuitetne varijante enzima za razgradnju biljne biomase pre svega kutina i lignina (Chen et al., 2020). Voluminozno aktivno mesto sposobno da smesti dugačke polimerne supstrate, prisustvo hidrofobnih aminokiselina u blizini aktivnog mesta i hidrofobni vezujući domeni su karakteristike koje definišu ove enzime (Amobonye et al., 2021, Nikolaivits et al., 2021).

Tabela 1- Najbolje izučeni enzimi i mikroorganizmi uključeni u razgradnju plastike (adaptirano iz Nikolaivits i saradnika).

Enzim	Mikroorganizam	Vrsta plastike	Rezultati	Referenca
Lakaza	*Rhodococcus ruber*	PE	2,5% gubitak mase	(Santo et al., 2013)
TfH	*Thermobifida fusca*	PET	54,2% gubitak mase	(Kleeberg et al., 2005)
TfCut2	*Thermobifida fusca*	PET	30% gubitak mase	(Furukawa et al., 2019b)
HiC	*Humicola insolens*	PET	97% gubitak mase	(Ronkvist et al., 2009)
LCC	Nepoznati miroorganizam	PET	95% gubitak mase	(Shirke et al., 2018)
Hidrohinon peroksidaza	*Azotobacter beijerinckii* HM121	PS	Smanjenje molekulskog broja sa 350 000 na 1 000	(Nakamiya et al., 1997)
PU esteraza	*Comamonas acidovorans* TB-35	PU	Oslobađanje monomera adipinske kiseline i 2,4-diaminotoluena	(Nomura et al., 1998)
Lipaza	*Cryptococcus* MTCC 5455	PU	96% gubitka mase	(Thirunavukarasu et al., 2015)

Prema podacima iz baze podataka enzima aktivnih na plastici (PAZy - The Plastics-Active Enzymes Database; https://pazy.eu) do sada je opisano i biohemijski okarakterisano svega 205 enzima sposobnih da razgrađuju različite vrste plastike (Buchholz et al., 2022). U pogledu biorazgradivosti plastični polimeri mogu da se podele u dve grupe zavisno od veza koje sačinjavaju okosnicu polimera. Prva grupa, kojoj pripadaju PE, PP, PVC i PS, predstavlja nehidrolizabilne polimere čija se okosnica sastoji od visoko stabilnih C-C veza koje ne podležu hidrolizi. Stoga, identifikovana su samo četiri enzima koja mogu da razgrade PE (Sanluis-Verdes et al., 2022, Zampolli et al., 2023). Enzimi sposobni da direktno razgrađuju PP, PVC i PS nisu do sada

identifikovani zbog čega se smatra da biotehnološka razgradnja ovih polimera trenutno nije primenljiva i većina napora usmerena je na drugu grupu polimera. Ta grupa, koju prevashodno čine PET i PU, su hidrolizabilni ili heteroatomski polimeri čija se okosnica sastoji od estarskih i uretanskih veza koje su znatno podložnije enzimskoj razgradnji (Chow et al., 2022). Do danas, ukupno su okarakterisana 122 enzima sa aktivnošću na PET i PU među kojima je i jedini enzim koji se koristi za razgradnju plastike na industrijskoj skali (Tournier et al., 2020). Zbog toga je u okviru ove doktorske disertacije akcenat bio na biotehnološkoj razgradnji hidrolizabilnih polimera PET i PU. **Tabela 1** prikazuje neke od najbolje proučenih enzima i mikroorganizama za razgradnju plastike, opisanih do 2021, gde se jasno uočavaju razlike između efekata enzima na gubitak mase PET i PU, u odnosu na PE.

1.3.1. Mikroorganizmi uključeni u razgradnju poli(etilentereftalata)

PET je polimer koji se dobija reagovanjem tereftalne kiseline[3] (TPA) i etilen-glikola[4]. Najčešće se koristi za proizvodnju plastičnih boca i u tekstilnoj industriji, a kao hidrolizabilni polimer sa najvećom godišnjom produkcijom ujedno je i najbolje izučen sa stanovišta biotehnološke razgradnje (Nikolaivits et al., 2021). Polimerni lanci PET se sastoje od visoko struktuiranih kristaliničnih regiona i nestrukturanih amorfnih regiona. Na temperaturama iznad 65°C amorfni regioni postaju fleksibilni i pristupačniji biokatalizatorima, shodno tome termostabilni biokatalizatori su poželjni pri razgradnji PET (Wei and Zimmermann, 2017). Gljive i aktinomicete su se pokazale kao vrlo efikasni biokatalizatori u razgradnji PET. Predstavnici roda *Thermobifida*, a naročito *T. fuca*, *T. cellulosilytica*, *T. alba* i *T. halotolerans*, se ističu u razgradnji PET materijala (Carr et al., 2020). Osim toga, pojedini sojevi *Bacillus subtilis* (Ribitsch et al., 2011), *Humicola insolens* (Ronkvist et al., 2009), *Pseudomonas aestusningri* (Bollinger et al., 2020) i *Streptomyces scabei* (Jabloune et al., 2020) su takođe prijavljeni da razgrađuju PET. Upotrebom ovih sojeva dolazi do modifikacija na površini polimera uz oslobađanje monomera, međutim, gubitak mase retko prelazi nekoliko procenata, a temperature na kojima ove bakterije optimalno rastu su znatno ispod temperatura neophodnih za efikasnu razgradnju PET (Kawai et al., 2019)

Najveći odjek u naučnoj javnosti izazvao je pronalazak bakterije *Ideonella sakaiensis* 2016. godine (Yoshida et al., 2016). Ova bakterija, izolovana sa površine otpadnih PET boca, može da koristi PET kao jedini izvor ugljenika i energije na temperaturi od 30°C. Razgradnja i asimilacija PET od strane *I. sakaiensis* postiže se dejstvom dva enzima: 1) vanćelijske depolimeraze (IsPETaza) koja razgrađuje polimer do (2-hidroksietil)-tereftalat (MHET) koji kasnije, dejstvom 2) unutarćelijskog enzima MHETaze, biva inkorporiran u metabolizam bakterije (Yoshida et al., 2016). Međutim, niske stope razgradnje, u smislu ukupne konverzije PET polimera u monomere (< 0,1%), učinile su upotrebu ove bakterije kao biokatalizatora neisplativom. Vodeći se time, istraživanja ka upotrebi celih mikroorganizama išla su u smeru povećavanja katalitičke aktivnosti sojeva primenom genetičkog inženjerstva. Konstruisani su *Bacillus* sojevi koji efikasno eksprimiraju različite PETaze i razgrađuju PET (Wang et al., 2020, Huang et al., 2018). Takođe,

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[3] IUPAC- benzen-1,4-dikarboksilna kiselina
[4] IUPAC- 1,2-etandiol

sojevi kvasca *Yarrovia lipolytica* konstruisani su da efikasno razgrađuju PET i koriste monomere nastale prilikom razgradnje (da Costa et al., 2020, Kosiorowska et al., 2022). Modifikacija soja kvasca *Pichia pastoris* GS115 da na površini ćelije ko-eksprimira PETazu iz *I. sakaiensis* i hidrofobin iz filamentozne gljive *Schizophyllum commune*, protein koji omogućava adheziju za hidrofobne površine, omogućila je povećanje katalitičke aktivnosti 328 puta. Ovaj sistem dostigao je 10,9% konverzije visoko kristaliničnog PET u monomere naspram oko 0,021% postignute sa ekvivalentnom koncentracijom prečišćene IsPETaze (Chen et al., 2022). Bez obzira na relativno veliki broj identifikovanih mikroorganizama i razvoj kompleksnih rekombinantnih sojeva, potraga za još efikasnijim biokatalizatorima za razgradnju PET se nastavlja.

1.3.2. Enzimi uključeni u razgradnju poli(etilentereftalata)

Usled nedostatka postupaka u kojima se koriste cele ćelije kao biokatalizatori, što se ogleda u ograničenoj sekreciji enzima, uskom opsegu temperatura na kojima dolazi do razgradnje PET i otežanog prečišćavanja proizvoda reakcije, pažnja je sve više usmerena na enzime koji hidrolizuju PET (PETaze) (Kawai et al., 2020). U prilog navedenom govori činjenica o trenutno 95 opisanih različitih PETaza i još nekoliko optimizovanih varijanti dobijenih proteinskim inženjerstvom (Buchholz et al., 2022). Iako je već pomenuti enzim IsPETaza vrlo specifičan za PET kao supstrat, ukupna stopa konverzije polimera u monomere je vrlo niska i ne prelazi 1% za 18 h na 30°C, a na temperaturama preko 40°C dolazi do potpunog gubitka enzimske aktivnosti (Yoshida et al., 2016). Usled povećane termostabilnosti i mogućnosti da hidrolizuju PET na visokim temperaturama enzimi TfCut1, TfCut2 i TfH iz različitih *Thermobifida* sojeva i HiC iz *H. insolens* pokazali su se kao znatno efikasniji u razgradnji PET uz oslobađanje MHET i TPA (Carr et al., 2020). Međutim, najpotentnije PETaze i ujedno PETaze sa najvećom termostabilnošću nisu izolovane iz mikroorganizama koji razgrađuju PET već iz nepoznatih mikroorganizama pretraživanjem metagenomskih biblioteka. Dva prirodna enzima sa najvišim stopama razgradnje, izolovana korišćenjem metagenomske DNK komposta, su LCC (eng. *Leaf Compost Cutinase*) i PHL7 PETaze (Sulaiman et al., 2012, Sonnendecker et al., 2022). Tako PHL7 u potpunosti razgrađuje amorfne PET filmove oslobađajući 91 mg/h TPA, a temperatura topljenja ovog enzima bila je 84°C, znatno iznad temperatura potrebnih za efikasnu razgradnju PET (Sonnendecker et al., 2022).

Kako bi se dodatno povećala aktivnost i termostabilnost PETaza pribegavalo se različitim pristupima. Dodavanjem jona Ca^{2+} i Mg^{2+} u rastvor moguće je povećati termostabilnost PETaza i do 15°C, ujedno pojačavajući razgradnju PET (Then et al., 2015). Takođe, pokazano je da anjonski i katjonski deterdženti mogu pomoći u adsorpciji PETaza na površinu plastike povećavajući efikasnost razgradnje i do 120 puta (Furukawa et al., 2018, Furukawa et al., 2019a). Efikasnost enzimske razgradnje PET je dodatno povećana korišćenjem kombinacija enzima. Karboksilesteraze (EC 3.1.1.1) i MHETaza korišćene su zajedno sa različitim PETazama kako bi time što hidrolizuju oligomere i monomere kao što su bis(2-hidroksietil)-tereftalat (BHET) i MHET smanjile inhibiciju proizvodima i tako povećale efikasnost PETaza (Mrigwani et al., 2022, Barth et al., 2016). Proteinskim inženjerstvom je takođe povećana termostabilnost i smanjena inhibicija proizvodima kod PETaza. Nekoliko unapređenih varijanti IsPETaze dobijeno je

proteinskim inženjerstvom koje je uključivalo racionalni dizajn i primenu mašinskog učenja (Lu et al., 2022, Son et al., 2019). Takođe, enzim LCC^{ICCG} , varijanta LCC sa samo 4 aminokiselinske zamene, sposobna je da razgradi 90% PET za samo 10 h oslobađajući TPA koja se može koristiti za ponovnu proizvodnju PET. Uzevši u obzir odlične katalitičke performanse i visoku stabilnost firma Carbios (Francuska; www.carbios.com) je iskoristila LCC^{ICCG} kao prvi enzim za razgradnju PET na industrijskoj skali (Tournier et al., 2020).

1.3.3. Mikroorganizmi uključeni u razgradnju poli(uretana)

Reagovanjem različitih diizocijanata i poliola moguće je dobiti polimere raznovrsnih karakteristika kolektivno nazvanih poli(uretanima) (PU). Zajednička karakteristika ovih polimera je uretanska (karbamatna) veza (Liu et al., 2021a). PU su šesta najčešća vrsta plastike koja se koristi u proizvodnji sunđera, dušeka, obuće, u građevinskoj i auto-moto industriji (Liang et al., 2021). Za razliku od PET koji ima jasno definisanu strukturu, PU pored uretanske veze u okosnici polimera često sadrže i druge vrste hemijskih veza. Najčešće vrste PU su poliestarski PU i polietarski PU koji uz uretansku sadrže i estarske i etarske veze, respektivno. Generalno, poliestarski PU pokazali su se pogodnijim za mikrobiološku razgradnju u odnosu na polietarske PU i stoga je većina istraživanja usmerena ka poliestarskom tipu (Magnin et al., 2020).

Neke od prvih naznaka da PU mogu biti razgrađeni od strane mikroorganizama objavljene su pre skoro četiri decenije kada je uočeno da se zemljišni mikroorganizmi mogu koristiti u ove svrhe (Bentham et al., 1987). Nakon toga, brojna istraživanja pokazala su da zajednice različitih mikroorganizama mogu da razgrađuju različite vrste PU (Vargas-Suárez et al., 2021). Međutim, niske stope razgradnje i vreme inkubacije u rasponu od nekoliko meseci pa i do dve godine ukazali su na potrebu za pronalaženjem pojedinačnih sojeva odgovornih za ovu aktivnost. Više bakterijskih sojeva koji pre svega pripadaju rodovima *Pseudomonas*, *Bacillus*, *Acinetobacter* i *Arthrobacter* prijavljeni su kao PU razgrađivači, a među gljivama dominantni rodovi su *Cladosporium*, *Alternaria* i *Aspergillus* (Magnin et al., 2020). Sojevi roda *Pseudomonas* su najbolje proučeni, sa preko 10 prijavljenih sojeva i nekoliko okarakterisanih enzima uključenih u razgradnju PU (Buchholz et al., 2022). *Commamonas acidovorans* soj TB-35 je jedan od retkih mikroorganizama koji u potpunosti razgrađuje PU filmove uz oslobađanje 2,4-diaminotoluena (Nakajima-Kambe et al., 1997, Nakajima-Kambe et al., 1995). *B. subtilis* MZA-75 i *P. aeruginosa* MZA-85 takođe mogu da razgrađuju PU ali i da mineralizuju oslobođene intermedijere razgradnje (Shah et al., 2013a, Shah et al., 2013b). Iako je broj opisanih mikroorganizama koji razgrađuju PU veći u odnosu na one koji razgrađuju druge polimere, bitno je napomenuti da mali broj ovih sojeva zapravo razgrađuje uretanske veze u polimeru koje su se pokazale vrlo otpornim na dejstvo mikroorganizama. Usled kompleksne strukture PU vrlo često dolazi do razgradnje samo dela polimera koji sadrži estarske veze, dok ostatak polimera, koji sadrži uretanske veze, ostaje netaknut (Biffinger et al., 2015). Zbog toga se mikroorganizmi koji razgrađuju i poliestarske i polietarske PU smatraju znatno primamljivijim u odnosu na one koji razgrađuju samo jednu vrstu PU s obzirom da razgradnja potiče od hidrolize uretanskih veza polimera. Jedan od retkih primera razgradnje polietarskih PU je upotreba konzorcijuma sastavljenog od *Cladosporium*, *Aspergilus* i

Penicillium sojeva. Nakon 21 dan inkubacije ovaj konzorcijum postizao je između 10% i 65% smanjenja mase polietarskog PU uz potvrdu razgradnje uretanske veze (Álvarez-Barragán et al., 2016).

1.3.4. Enzimi uključeni u razgradnju poli(uretana)

Enzimska razgradnja PU pre svega podrazumeva dejstvo esteraza, amidaza (EC 3.5) i proteaza (EC 3.4) (Magnin et al., 2020). Od 27 enzima do sada okarakterisanih da deluju na PU većina su esteraze koje deluju na estarske veze unutar PU (Buchholz et al., 2022). PudA iz *C. acidovorans*, PueA, PueB i PulA iz vrsta roda *Pseudomonas* pokazuju odličnu aktivnost na model supstratima kao što je Impranil ali nemaju skoro nikakvu aktivnost na drugim vrstama PU (Liu et al., 2021a). Znatno više uspeha u razgradnji različitih vrsta PU postignuto je upotrebom esteraza koje ujedno mogu da razgrađuju i PET. LCC i TfCut2 uspešno razgrađuju PU na temperaturi od 70°C, međutim, ukupno smanjenje mase posle 200 h reakcije bilo je svega oko 4% (Schmidt et al., 2017). Zbog nedovoljne efikasnosti esteraza, amidaze, za koje je dokazano da mogu hidrolizovati uretansku vezu, podjednako su okupirale interesovanje istraživača. Amidaza iz *Nocardia farcinica*, ranije potvrđena da razgrađuje i monomere najlona, pokazala se kao obećavajući biokatalizator za razgradnju PU. Nadograđivanjem ovog enzima sa dodatnim polimer-vezujućim domenom PHA depolimeraze iz *Alcaligenes faecalis* poboljšana je adsorpcija i katalitička aktivnost prema polietarskom PU uz specifičnu hidrolizu uretanske veze (Gamerith et al., 2016). Integrisani hemijsko-biološki sistemi za razgradnju PU koji uključuju upotrebu amidaza su takođe predloženi. Amidaze UMG-SP-1, UMG-SP-2 i UMG-SP-3, identifikovane korišćenjem metagenomske DNK iz zemljišta zagađenog sa PU, su korišćene za razgradnju molekula, koji sadrže uretansku vezu, zaostalih nakon hemijske depolimerizacije PU. Upotrebom ovog sistema omogućeno je dobijanje smeše monomera koji se mogu dalje prečišćavati (Branson et al., 2023).

Uzimajući u obzir kompleksnu strukturu PU jasno je da upotreba pojedinačnih enzima nije adekvatno rešenje za ovu grupu polimera. Magnin i saradnici razvili su sistem koji je uključivao sinergističko dejstvo esteraze i amidaze kako bi postigli razgradnju poliestarskog PU uz uspešno prečišćavanje monomera koji se mogu koristiti za ponovnu sintezu PU (Magnin et al., 2019b). Oksidoreduktaze se takođe smatraju obećavajućom alternativom za enzimsku razgradnju PU. Proteomska analiza marinske vrste *Bacillus velezenis* GUIA pokazala je da je ključni enzim u razgradnji poliestarske PU disperzije oksidaza Oxr-1 koja je pored PU razgrađivala i poli(butilen-adipat-*co*-tereftalat) (biorazgradiva alternativa za PET) (Gui et al., 2023). Takođe, komercijalno dostupna lakaza iz *T. versicolor* identifikovana je kao još jedna oksidoreduktaza koja je uspešno razgrađivala kako poliestarske tako i polietarske PU (Magnin et al., 2021). Važno je napomenuti da neka istraživanja impliciraju da čak i dekarboksilaze, klasa enzima do sada zanemarena u istraživanjima razgradnje plastike, učestvuju u razgradnji PU zajedno sa različitim hidrolazama (Bhavsar et al., 2023). Prema do sada sprovedenim istraživanjima enzimska razgradnja PU se pokazala kao znatno veći izazov i nesumnjivo će uključivati više od jedne klase enzima.

1.4. Bioplastika

Zamena teško razgradivih materijala materijalima koji su održivi i biorazgradivi, vodeći računa o funkcionalnosti, jedna je od strategija smanjenja akumulacije plastičnog otpada (odeljak 1.2). Prema preporukama Međunarodne unije za čistu i primenjenu hemiju (IUPAC), bioplastika se definiše kao polimer izveden iz biomase ili sastavljen od monomera poreklom iz biomase koji se u nekom trenutku proizvodnje može ukalupiti u određen oblik (Vert et al., 2012). Češće, bioplastika se koristi kao krovni termin za vrlo heterogenu grupu polimera koji moraju da ispune jedan od dva kriterijuma. Moraju biti biorazgradivi, što podrazumeva potpunu razgradnju od strane sredinskih mikroorganizama u razumnom vremenskom periodu, ili moraju biti u celosti ili delimično sastavljeni od ugljenika iz bioloških izvora (Nanda et al., 2022). Imajući u vidu vrlo pogodne fizičke i hemijske karakteristike, koje pariraju polimerima dobijenih iz neobnovljivih resursa, bioplastika nastoji da zameni konvencionalnu plastiku kao održiva i ekološki isplativija alternativa (Thakur et al., 2018). Trenutno, bioplastika predstavlja svega 1,5% ukupne globalne proizvodnje plastike (PlasticsEurope, 2022), međutim, predviđa se da će se tržište bioplastike utrostručiti u sledećih nekoliko godina (Degli Esposti et al., 2021). Neke od najčešće korišćenih bioplastika su poli(hidroksialkanoati) (PHA), poli(kaprolakton) (PCL), poli(butilen-sukcinat) (PBS), poli(mlečna kiselina) (PLA), poli(vinil-alkohol) (PVA) i različite mešavine polimera sa skrobom i celulozom (Jeremić et al., 2020). PHA predstavljaju porodicu prirodnih poliestara, proizvedenih od strane mikroorganizama kao citoplazmatski depo ugljenika i energije. Dužina monomera PHA diktira karakteristike polimera na osnovu čega se i dele na PHA kratkog (3 do 5 C atoma) i dugog niza (6-16 C atoma) (Chen, 2009). Poli(hidroksibutirat) (PHB) je prvi identifikovani PHA koji se odlikuje manjom propustljivošću za gasove i većom čvrstinom od konvencionalnih petrohemijskih plastika PP i PE dok je poli(hidroksioktanoat) (PHO) vrlo fleksibilan polimer. Tako, mešanjem PHA različitih dužina lanca moguće je proizvoditi polimere strogo definisanih karakteristika koji se mogu koristiti u različite svrhe (McAdam et al., 2020). Zajedno sa PLA i polimerima na bazi skroba PHA predstavljaju biorazgradive bioplastike biološkog porekla. Dok sa druge strane PCL i PBS predstavljaju najčešće biorazgradive polimere dobijene iz neobnovljivih resursa (Jeremić et al., 2020).

Iako bioplastika obećava kao materijal budućnosti, istraživači upozoravaju da biorazgradivost ovih polimera ne treba uzimati zdravo za gotovo. Bioplastike i mešavine bioplastika pokazale su se relativno otpornim na razgradnju u različitim sredinama *in vivo*, a većina ovih polimera ne zadovoljava kriterijume za biorazgradnju Međunarodne organizacije za standardizaciju (ISO) (Narancic et al., 2018). Bioplastični otpad može kontaminirati otpadne tokove drugih plastika što značajno otežava njihovo recikliranje (Nandakumar et al., 2021). Zbog ovih nedostataka neophodno je uporedo sa razvijanjem novih polimera razvijati i prilagodljive sisteme za sakupljanje i recikliranje bioplastike.

1.5. Biološka valorizacija plastičnog otpada

U cilju poboljšanja ekološke održivosti kako biotehnoloških tako i hemijskih strategija za rešavanje problema plastičnog otpada predložena je valorizacija ovog otpada u jedinjenja i materijale sa dodatom vrednošću- „upcycling" (odeljak 1.2) (Blank et al., 2020).

Proizvodi pirolize PS i PET direktno su iskorišćeni kao izvor ugljenika za proizvodnju bioplastike PHA od strane različitih sojeva *Pseudomonas*, postižući umerene do dobre prinose optimizacijom strategija fermentacije (Kenny et al., 2008, Nikodinovic- Runic et al., 2011). PE vosak dobijen pirolizom, sastavljen od alifatičnih ugljovodonika, takođe je iskorišćen za proizvodnju PHA uz prisustvo biosurfaktanata (Guzik et al., 2014). Skorije, razvijeni su sistemi za direktnu biološku konverziju PET u PHB primenom rekombinantnih sojeva *Yarrowia* i *Pseudomonas* (Liu et al., 2021b). Kao i za enzimsku konverziju PET u biorazgradive PU. Upotrebom termostabilne PETaze PET je depolimerizovan na TPA i etilen-glikol koji su dalje upotrebljeni kao izvor ugljenika za *P. umsongensis* GO16 modifikovane da sekretuje intermedijere sinteze PHA. Finalno, ovi intermedijeri iskorišćeni su kao polazna jedinjenja za hemijsku sintezu biorazgradivih PU (Tiso et al., 2021).

Koncept koji je privukao dosta pažnje je korišćenje bakterijskih konzorcijuma kako bi se valorizovao PET otpad. Dva soja *P. putida* konstruisana tako da jedan koristi TPA, a drugi etilen-glikol, pokazali su se kao znatno efikasniji i prilagodljiviji sistem za prevođenje PET hidrolizata u PHA i mukonsku kiselinu[5] koja se dalje može koristiti u sintezi nekoliko različitih polimera (Bao et al., 2023). Pored već pomenute upotrebe u ponovnoj sintezi PET i drugih polimera, produkti razgradnje PET mogu se koristiti kao reagensi za dobijanje novih jedinjenja. Upotrebom rekombinantnih sojeva *Escherichia coli* TPA je korišćena kao osnov za sintezu različitih jedinjenja, uključujući galnu kiselinu[6], pirogalol[7], katehol[8],vanilinsku kiselinu[9] i mukonsku kiselinu (Kim et al., 2019b). Produkti razgradnje PU takođe mogu biti iskorišćeni od strane mikroorganizama i pretvoreni u proizvode sa dodatom vrednošću. Sintetički konzorcijum mikroorganizama, sastavljen od rekombinantnih *Pseudomonas* sojeva, korišćen je za pretvaranje hipotetičke mešavine produkata razgradnje PU, adipinske kiseline[10], 1,4-butandiola, etilen-glikola i 2,4-diaminotoluena u ramnolipide, uz napomenu da je 2,4-diaminotoluen inhibirao rast većine članova konzorcijuma i morao biti ekstrahovan radi efikasnije proizvodnje (Utomo et al., 2020).

Pored gore navedenih primera, različite vrste gljiva iskorišćene su za biološku valorizaciju hemijski depolimerizovanih PE i PS. Smeše degradacionih produkata uspešno su konvertovane u

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[5] IUPAC- (2*E*,4*E*)-heksa-2,4-diendionska kiselina
[6] IUPAC- 3,4,5- trihidroksibezojeva kiselina
[7] IUPAC- 1,2,3- trihidroksibenzen
[8] IUPAC- benzen- 1,2- diol
[9] IUPAC- 4-hidroksi-3-metoksibenzoeva kiselina
[10] IUPAC- heksandionska kiselina

medicinski relevantne sekundarne metabolite mutilin[11], ergotionin[12], pleuromutilin[13], citreoviridin[14] i asperbenzaldehid[15] sa značajnim prinosima čime se proširuje spektar jedinjenja koji se može dobiti valorizacijom plastičnog otpada (Rabot et al., 2023b, Rabot et al., 2023a). Hemijski depolimerizovan PE takođe je korišćen za proizvodnju visoko specifičnih rekombinantnih proteina (zeleni fluorescentni protein i protein paukove mreže) sa prinosima od preko 10 mg/mL (Connor et al., 2023).

Trenutno svi pomenuti procesi predstavljaju samo dokaze koncepta biotehnološke konverzije u jedinjenja sa dodatom vrednošću na laboratorijskoj skali. Kako bi ovi pristupi zaista našli primenu na industrijskoj skali neophodno je obaviti detaljne analize životnog ciklusa (eng. *Life Cycle Assessment*) ovih procesa. Analiza životnog ciklusa podrazumeva sistematsku i sveobuhvatnu metodu za procenu ekološkog otiska proizvoda, procesa ili usluge tokom celog životnog ciklusa, od ekstrakcije sirovina do odlaganja. Ova metoda uključuje ocenu korišćenja resursa, potrošnje energije, oslobađanja toksičnih jedinjenja i drugih ekoloških aspekata kako bi se stekao uvid u održivost procesa (Horodytska et al., 2020). Ovakva vrsta analize identifikovala je niz problema u industrijskom procesu enzimske razgradnje PET kako bi se dobila TPA za ponovnu sintezu. Proces koji uključuje enzimsku depolimerizaciju pokazao se i do 17 puta štetniji po životnu sredinu u poređenju sa prečišćavanjem fosilnih resursa i dobijanja TPA na taj način (Uekert et al., 2022). Uprkos trenutnim upozorenjima o neodrživosti biološke razgradnje i valorizacije plastičnog otpada detaljne analize životnog ciklusa su neophodne kako bi poslužile kao osnov za razvijanje novih strategija. Uz adekvatno planiranje i analizu nesumnjivo je da će valorizacija otpada igrati veliku ulogu u obezbeđivanju ekonomske isplativosti strategija za rešavanje plastičnog otpada.

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[11] IUPAC- (1*S*,2*R*,3*S*,4*S*,6*R*,7*R*,8*R*,14*R*)-4-etenil-3,6-dihidroksi-2,4,7,14-tetrametiltriciklo[5.4.3.01,8]tetradekan-9-on

[12] IUPAC- (2*S*)-3-(2-sulfaniliden-2,3-dihidro-1H-imidazol-4-il)-2-(trimetilazaniumil)propanoat

[13] IUPAC- (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-etenil-5-hidroksi-4,6,9,10-tetrametil-1-oksodekahidro-3a,9-propanociklopenta[8]anulen-8-il hidroksiacetat

[14] IUPAC- 6-[(1*E*,3*E*,5*E*,7*E*)-8-[(2*S*,3*R*,4*R*,5*R*)-3,4-dihidroksi-2,4,5-trimetiloksolan-2-il]-7-metilokta-1,3,5,7-tetraenil]-4-metoksi-5-metilpiran-2-on

[15] IUPAC- 6-((*R*,3*E*,5*E*)-5,7-dimetil-2-oksonona-3,5-dienil)-2,4-dihidroksi-3-metilbenzaldehid

2. CILJ ISTRAŽIVANJA

Prekomerna proizvodnja i upotreba plastike kao i neadekvatni sistemi za njeno odlaganje, sakupljanje i recikliranje doveli su do nakupljanja plastičnog otpada u životnoj sredini koje je praćeno nizom negativnih posledica. Bez obzira na brojne napore da se problem reši, efikasne strategije za zbrinjavanje plastičnog otpada i dalje nedostaju. Biotehnološka razgradnja i valorizacija plastičnih polimera može predstavljati ekološki održiv, ali i ekonomski isplativ pristup koji je privukao interesovanje naučne javnosti i industrije.

Glavni cilj ove doktorske disertacije je identifikovanje i karakterizacija potencijalnih biokatalizatora, kako bakterijskih sojeva, tako i njihovih enzima koji se mogu upotrebiti u procesima razgradnje i valorizacije plastičnog otpada. Posebna pažnja posvećena je PET i PU, budući da spadaju u grupu najčešće korišćenih polimera, a u svojoj okosnici sadrže veze podložne enzimskoj hidrolizi.

U skladu sa opštim ciljem doktorske disertacije postavljeni su sledeći specifični ciljevi:

1. Utvrditi potencijal kolekcije mikroorganizama Grupe za eko-biotehnologiju i razvoj lekova (IMGGI) iz zagađenih i nezagađenih staništa za razgradnju plastike;

2. Ispitati mehanizam razgradnje PET od strane mikroorganizama koji su pokazali najveći potencijal za razgradnju;

3. Utvrditi koji enzimi odabranih sojeva su uključeni u razgradnju PET;

4. Ispitati mehanizam razgradnje PU od strane mikroorganizama koji su pokazali najveći potencijal za razgradnju;

5. Utvrditi koji enzimi odabranih sojeva su uključeni u razgradnju PU i efekat razgradnje na centralni metabolizam;

6. Uspostaviti sistem za pretvaranje različitih plastičnih polimera u jedinjenja sa dodatom vrednošću- poli(hidroksibutirat) i undecilprodigiozin.

3. NAUČNI RADOVI PROIZAŠLI IZ DOKTORSKE DISERTACIJE

1.1. A novel *Bacillus subtilis* BPM12 with high bis(2 hydroxyethyl)terephthalate hydrolytic activity efficiently interacts with virgin and mechanically recycled polyethylene terephthalate

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A novel *Bacillus subtilis* BPM12 with high bis(2 hydroxyethyl)terephthalate hydrolytic activity efficiently interacts with virgin and mechanically recycled polyethylene terephthalate

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ABSTRACT

Biotechnological treatment of plastic waste has gathered substantial attention as an efficient and generally greener approach for polyethylene terephthalate (PET) depolymerization and upcycling in comparison to mechanical and chemical processes. Nevertheless, a suitable combination of mechanical and microbial degradation may be the key to bringing forward PET upcycling. In this study, a new strain with an excellent bis(2 hydroxyethyl)terephthalate (BHET) degradation potential (1000 mg/mL in 120 h at 30 °C) and wide temperature (20-47 °C) and pH (5-10) tolerance was isolated from a pristine soil sample. It was identified as *Bacillus subtilis* BPM12 via phenotypical and genome analysis. A number of enzymes with potential polymer degrading activities were identified, including carboxylesterase BPM12CE that was efficiently expressed both, homologously in *B. subtilis* BPM12 and heterologously in *B. subtilis* 168 strain. Overexpression of this enzyme enabled *B. subtilis* 168 to degrade BHET, while the activity of BPM12 increased up to 1.8-fold, confirming its BHET-ase activity. Interaction of *B. subtilis* BPM12 with virgin PET films and films that were re-extruded up to 5 times mimicking mechanical recycling, revealed the ability of the strain to attach and form biofilm on each surface. Mechanical recycling resulted in PET materials that are more susceptible to chemical hydrolysis, however only slight differences were detected in biological degradation when BPM12 whole-cells or cell-free enzyme preparations were used. Mixed mechano/bio-degradation with whole-cells and crude enzyme mixes from this strain can serve to further increase the percentage of PET- based plastics that can enter circularity.

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1. Introduction

In the last decade, global plastic production reached over 360 million tons annually (Magalhães et al., 2021). Plastic pollution has become the focus of numerous scientific studies and industry lead-efforts (Laskar and Kumar, 2019). However, the solution for efficient large-scale plastic degradation/regeneration and recycling remains elusive. Further exaggerating the environmental impacts, plastic production uses about 8% of the world's fossil fuel resources, releasing greenhouse gasses and contributing to global warming (Samak et al., 2020). Europe is the leader in plastic recycling, with close to 29 million tons of post-consumer plastics collected out of 55 million tons produced in 2020. However, only 34.6% of collected materials were recycled while 42% were incinerated for energy recovery and 23.4% were landfilled (PlasticsEurope, 2021). Hence, additional research is needed to develop efficient strategies for tackling the problem of plastic waste accumulation and its adverse effects on the environment and health.

Polyethylene terephthalate (PET) is a synthetic polyester with a heteroatomic backbone made by reacting ethylene glycol (EG) and terephthalic acid (TPA). It represents 8.4% (*w/w*) of the total plastic produced and it is mainly used for beverage bottles (Kosiorowska et al., 2022). Efficient PET recovery of high-grade PET waste, such as beverage bottles, has been developed to provide "clean" and "high purity" PET waste streams for recycling. Recovered PET can undergo re-extrusion (enabling recovery of uncontaminated PET scraps in manufacturing plants), mechanical recycling (reprocessing PET into granules via extrusion processes yielding PET with reduced performances), chemical recycling (a variety of chemical processes for the depolymerization of PET and subsequent repolymerization into new polymers) and energy recovery (Benyathiar et al., 2022). Recycled PET is a commodity with many end uses, for the benefit of society and the environment. Traditionally mechanical recycling is the most widely used method of PET recycling and its application is likely to increase in the following years due to its low energy consumption and absence of use of hazardous chemical reagents (Suzuki et al., 2022). Rules have been adopted to ensure that recycled plastic can be safely used in food packaging and contribute to the overall sustainability towards achieving the objectives of the Circular Economy Action Plan (Commission, 2022). Processing including solid state polycondensation (SSP) can increase molecular weight and achieve parameters required to produce food contact approved PET according to regulation (EU) No 10/2011 and No 64/201. On the other side, chemical recycling can be applied to a wider range of mixed plastic waste but in many cases carries the burden of involving additional harmful chemicals and costs in the processes. Through chemical recycling, even multilayer colored PET plastic waste can be depolymerized into its main building blocks, allowing repolymerization following arduous purification or in the case of polyolefins, liquefaction through a thermo-chemical process can be used for conversion into products similar to crude oil (Ragaert et al., 2017). Therefore, milder conditions for chemical recycling and combination with other lower carbon means of polymer depolymerization should be explored.

In contrast to mechanical and chemical recycling, biocatalysis has emerged as an environmentally friendly and efficient approach for PET recycling (Nguyen et al., 2023; Wei and Zimmermann, 2017). The ester bonds which make up the backbone of the polyester polymer are susceptible to enzymatic degradation via hydrolysis by a number of enzymes with esterase activities, including PETases, lipases, cutinases and carboxylesterases (Jaiswal et al., 2020; Nikolaivits et al., 2022). The highly hydrophobic PET polymer is broken down into a variety of largely soluble oligomer degradation intermediates during enzymatic degradation. Through a series of endo- and exo- cleavages of ester bonds, products such as bis(2 hydroxyethyl)terephthalate (BHET), mono(2-hydroxyethyl)terephthalate (MHET), TPA and EG or their mixtures can be obtained (Mrigwani et al., 2022). TPA can then be purified and reused for PET manufacturing thus providing a route for a circular economy (Tournier et al., 2020). PET hydrolysis products can also be upcycled to commodity chemicals (Kim et al., 2019), polyhydroxyalkanoates (Kenny et al., 2008; Tiso et al., 2021), or even lycopene (Diao et al., 2023).

Research into the biological degradation of PET has revealed numerous bacterial and fungal strains harboring PET-degrading enzymes with over 8000 putative ortholog PETases identified in the genome databases (Gambarini et al., 2021). Highly efficient enzymes such as IsPETase from the bottle-dwelling bacterium *Ideonella sakaiensis* (Yoshida et al., 2016), or leaf-branch compost cutinase (LCC) identified through functional metagenomic screening (Sulaiman et al., 2012) have been reported to hydrolyze PET. Although biocatalytic processes are generally considered environmentally friendly, an in-depth life cycle assessment (LCA) of the enzymatic PET recycling revealed that it has up to 17 times worse environmental impact than manufacturing PET from virgin monomers (Uekert et al., 2022). To make biocatalytic and biotechnological processes truly advantageous further optimization work is needed.

The usefulness of PET oligomer degrading enzymes has been demonstrated in systems combining chemical and biological degradation, as well. PET degradation products obtained by glycolysis were efficiently converted to TPA by the addition of the Bs2Est esterase from *Bacillus subtilis* and subsequently transformed to catechol by an engineered *Escherichia coli* strain (Kim et al., 2021). Therefore, when searching for novel strains with PET degrading ability, it is important to search for enzymes that show high activity towards PET oligomers and other partial degradation products. This opens up the possibility of combining mechano- and green chemical depolymerizations with biocatalysis, as partially degraded polymers are still preferred substrates for enzymes and microorganisms.

In this study, an effort has been made to: (i) isolate and characterize a new bacterial strain capable of efficient degradation of BHET and other PET degradation intermediates, (ii) determine the enzymes responsible for this activity through genome analysis and expression of selected ones; (iii) and explore and evaluate how this strain can be utilized in biocatalytic degradation of multiple times extruded PET polymers mimicking the mechanical recycling process.

2

2. Materials and methods

2.1. Chemicals and reagents

Virgin polyethylene terephthalate (V-PET) resin in granulated form was purchased from Alpek Polyester UK Ltd. (Lazenby, UK). Components used to prepare media for bacterial growth were supplied by Acros Organics (Geel, Netherlands). Plastic monomers and polymers terephthalic acid (TPA), bis(2 hydroxyethyl) terephthalate (BHET), polycaprolactone diol (PCL) were purchased from Sigma Aldrich (Hamburg, Germany), Impranil DLN SD and Impranil DL 2077 from Covestro (Leverkusen, Germany). PET monomers and oligomers (1MER (1-(2-hydroxyethyl)-4-methylterephthalate), 1.5MER (ethylene glycol bis(methyl terephthalate), 2MER (methyl bis(2-hydroxyethyl terephthalate)) and 3MER (methyl tris(2-hydroxyethyl terephthalate)) (Fig. S1) were previously synthesized and described (Djapovic et al., 2021). Analytical grades of sodium hydroxide (98%), ethylene glycol (99%) (EG), kanamycin, and other salts and solvents were obtained from Sigma Aldrich (Hamburg, Germany). Restriction enzymes and lysozyme were purchased from Promega (Madison, USA).

2.2. Isolation, identification, and morphology of strain BPM12

Strain BPM12 was isolated from soil with limited vegetation (Maganik, Montenegro, with coordinates: 42°43′54″N 19°17′02″E) using standard nutrient rich LB agar (Luria Bertani agar, 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl and 15 g/L agar) as a part of the effort to make a diverse in-house microbial collection. This collection was used for a variety of bioprospecting studies including plastic degradation. The growth of BPM12 was assessed and compared to *B. subtilis* 168 Marburg (MoBiTec, Goettingen, Germany) on MSF (Mannitol soy flower, 20 g/L soy flower, 20 g/L mannitol and 20 g/L agar), MSM (Minimal 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, 15 g/L agar and 20 g/L glucose as carbon source) and LB plates at 30 °C. Growth temperature (15–47 °C) and pH (pH 2–12, adjusted with HCl and NaOH) ranges were tested in LB broth. 1% of overnight culture in LB was used as inoculum and the growth was monitored by measuring the absorbance at 600 nm (Ultrocpec 3300pro, Amersham Biosciences, Amersham, UK) after 24 h of incubation in an orbital shaker at 180 rpm (MaxQ 6000, Thermo Fisher Scientific, Waltham, USA).

The ability to ferment different carbohydrate substrates was assessed using an API 50 CHB test kit (bioMerieux, Marcyl'Etoile, France) and hemolytic activity was tested using blood agar. For further identification of BPM12, 16S rDNA was amplified via PCR (FastGene TAQ PCR Kit, Nippon Genetics, Düren, Germany) using standard 1492R and 27F primers and sequenced by Macrogen Europe BV (Amsterdam, Netherlands). The strain was identified using BLAST (Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/Blast.cgi), while the sequences were analyzed, and the phylogenetic tree was constructed using Mega 7 program (Molecular Evolutionary Genetics Analysis; www.megasoftware.net/home) and Maximal likelihood method.

The morphology of the cells was assessed using fluorescent microscopy. An overnight culture of BPM12 from LB medium was collected by centrifugation (10 min at 5000 g, Eppendorf 5804 centrifuge, Hamburg, Germany), washed and resuspended using phosphate-buffered saline (PBS) (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4; pH 7.2). Cells were fixed with paraformaldehyde and stained with 10 µg/mL of CI-TO-5 dye dissolved in PBS for 30 min at room temperature in the dark (Kurutos et al., 2020). The cells were visualized using an Olympus BX51 (Applied Imaging Corp., San Jose, USA) fluorescent microscope under 100000 × magnification.

2.3. Assessment of B. subtilis BPM12 plastic degrading potential

The plastic degrading potential of this bacterial strain was assessed using MSM agar plates supplemented with different plastic polymers and monomers as the sole carbon source applying previously described methodology (Molitor et al., 2020). The following substrates were used: TPA 10 g/L, BHET 10 g/L, PCL 6 g/L, Impranil DLN SD 6 g/L and Impranil DL-2077 9 g/L. The polymers and monomers were sonicated (Soniprep 150, MSE (UK) Ltd., Lindon, UK) for 10 min at 10 kHz before adding to the medium to obtain a stable suspension. The plates were incubated for 10 days at 30 °C and the formation of clearing zones was considered as a positive result.

2.3.1. Biotransformation of PET-related model substrates

BHET and four PET-related model substrates (Djapovic et al., 2021) were used to further investigate the PET degrading potential of strain BPM12. Reactions were carried out in 3 mL of MSM medium with 1 mg/mL of PET-related model substrates (added from stock solutions of 30 mg/mL in methanol). Bacterial cells from fresh LB agar plates were scraped with inoculating loop and resuspended in MSM medium to make resting cells suspension of 20 mg wet weight per mL and 100 µL of the cell suspension was added to all reactions. Reactions were incubated for 5 days at 30 °C and 150 rpm (MaxQ 6000, Thermo Fisher Scientific, Waltham, USA).

To monitor the reaction progress, reaction products were extracted from 100 µL using ethyl acetate and analyzed using thin layer chromatography (TLC) on alumina plates with 0.25 mm silica layer (TCL Silica gel 60 F_{254}, Sigma Aldrich (Hamburg, Germany). The solvent system was chloroform/methanol (8:2) and visualized using UV light at 254 nm (Camag UV Lamp, Camag, Wilmington, USA).

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2.3.2. High-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) analysis of biotransformation products

Samples were prepared by adding 1 μL of 6 M HCl to 1 mL of the reaction aliquot, vortexed and centrifuged for 10 min at 12000 g (Eppendorf Centrifuge 5417 R, Hamburg, Germany). The supernatant was filtered through 0.2 μm syringe filters. An UltiMate 3000 HPLC (Thermo Fisher Scientific, Waltham, USA) equipped with a Eurospher II 100-3 C18 A 150 × 4.6 mm (Knauer, Berlin, Germany) column was used for HPLC analysis. The mobile phase consisted of 20% (*v/v*) acetonitrile and 80% (*v/v*) 2.5 mM sulfuric acid in ultrapure water at a flow rate of 0.8 mL/min under isocratic conditions. Detection of reaction products was carried out at 241 nm. The total run time was 25 min.

The exact masses of PET-related model substrate degradation products were confirmed using the same HPLC method (at a flow rate of 0.3 mL/min) and a TSQ Fortis™ Plus triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, USA) equipped with an H-ESI source in mixed scan mode and single ion monitoring (SIM) scan type. The ionization parameters were: 4500 V positive spray voltage, 2600 V negative spray voltage, 50 arbitrary units (arb) sheath gas flow rate, 10 arb aux gas flow rate, 325 °C ion transfer tube temperature and 350 °C vaporizer temperature.

2.4. BPM12 genome sequencing, annotation and analysis

A 350 bp insert size library was prepared and sequenced in paired-end mode (read length, 150 bp) by Novogene Europe on a NovaSeq 6000 (Illumina, San Diego, USA) instrument and a total of 4,607,303 paired reads were generated. Raw reads were preprocessed with TrimGalore v0.6.5 and cutadapt v2.9 (Martin, 2011). The Illumina adapter sequences were removed (with a stringency of 3), bases with a quality score less than 10 were trimmed and reads smaller than 100 bases or with no pair were discarded. *De novo* genome assembly was performed with Spades v3.13.0 (Bankevich et al., 2012). Genome completeness was assessed with BUSCO v5.1.2 using the Bacillales single-copy orthologs from OrthoDB v10 (Manni et al., 2021). Strain BPM12 was phylogenetically classified with the Genome Taxonomy Database Toolkit v2.0.0 (Chaumeil et al., 2020) against the GTDB release 207.

Gene prediction and functional annotation were performed with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP, release 2022-10-03) (Li et al., 2021). Protein sequences were searched against the InterPro database with InterProScan v5.59-91.0 (Jones et al., 2014) and for signal peptides with SignalP v6.0 (Teufel et al., 2022). This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession .

The proteome of BPM12 was searched for homologs of biochemically characterized plastic-active enzymes from the PAZy database (Buchholz et al., 2021) with BLAST. The alignments were filtered for protein sequence identity > 40% and for > 70% alignment coverage of both the template and the target sequence. Next, the proteomes of 3 other *Bacillus* strains with reported activity on PET from RefSeq were gathered and clustered into homologous groups with the protein sequences of BPM12 using the Get_Homologues software (Contreras-Moreira and Vinuesa, 2013) with the bidirectional BLAST best-hit option and default settings. The three *Bacillus* strains were: *Bacillus* sp. AIIW2, *B. albus* PFYN01 and *B. thuringiensis* C15 (accession numbers: GCF_009932115.1, GCF_004153665.1 and GCF_004153515.1, respectively).

2.5. Overexpression and deletion of BPM12 carboxylesterase

BPM12 carboxylesterase (*bpm12CE* gene) was amplified via PCR (FastGene TAQ PCR Kit, Nippon Genetics, Düren, Germany) from BPM12 genomic DNA using BPM12CEF and BPM12CER primers containing the HindIII and BamHI restriction sites (Table S1). The amplicon was cloned into pGEM T-Easy (Promega, Madison, USA) vector, clones were confirmed via PCR amplification of the *Bpm12CE* gene and the appropriate restriction digest. The *bpm12CE* gene was then transferred to the pBE-S vector (Takara Bio, Shiga, Japan) using the HindIII and BamHI restriction enzymes. The pBE-S + *bpm12CE* plasmid constructs were used for the transformation of *B. subtilis* 168 Marburg (MoBiTec, Goettingen, Germany) cells and *B. subtilis* BPM12 using electroporation following the previously developed protocol (Yi and Kuipers, 2017). To create a *bpm12CE* knockout mutant a fusion gene consisting of two 1.5 kb flanking regions of *bpm12CE* and spectinomycin resistance gene was constructed using a set of primers shown in Table S1 and the NEBuilder HiFi DNA Assembly kit (New England Biolabs, Ipswich, USA). The fragment was used to transform *B. subtilis* BPM12 cells and *bpm12CE* was exchanged with the spectinomycin resistance gene. The knockout mutants were selected using spectinomycin 100 μg/mL and were confirmed via PCR using appropriate primers.

Growth and clearance of BHET by recombinant strains was assessed on LB and MSM agar plates containing 5 g/L of BHET and kanamycin 50 μg/mL or spectinomycin 100 μg/mL and agar 15 g/L. Recombinant strains were also used in biotransformation reactions of BHET as described previously.

The esterase activity of the recombinant strains was tested using *p*-nitrophenyl butyrate (pNPB) as substrate (Jaeger and Kovacic, 2014). The assay reagent was prepared by dissolving 0.088 g/L pNPB in 20 mM Na-phosphate buffer (pH 7.2) with 0.17 g/L SDS and 10 g/L Triton-X-100. Protein preparations (50 μL) were added to 150 μL of the reagent and incubated for 5 min at 30 °C. The reaction was monitored at 410 nm (Epoch Microplate Spectrometer, BioTek, Winooski, USA). The protein concentration of samples was determined using Bicinchoninic Acid Kit for Protein Determination (Sigma Aldrich, Hamburg, Germany) and adjusted to 500 μg/mL.

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2.6. Interaction of BPM12 with PET materials

2.6.1. Preparation of multiple mechanically recycled PET films

V-PET pellets were dried for 6 h at 150 °C in a Universal Oven U (Memmert GmbH, Schwabach, Germany) under forced ventilation until moisture content was below 0.005%. Mechanical recycling of PET was simulated by means of hot melt extrusion (Fig. S2). A bench-top Prism™ twin-screw extruder (Thermo Electron GmbH, Karlsruhe, Germany) was used to produce the samples used in this study. The diameter of the screws used was 16 mm, with a 25/1 length-to-diameter ratio, at a screw speed of 50 rpm. A temperature profile of 70, 230, 250, 250 and 250 °C for the five temperature control zones, followed by a 3-roll calendar configuration used to form films. The virgin resin was extruded and reprocessed to produce the following materials: V-PET (0 recycling cycles), R²-PET (2 recycling cycles), and R₅-PET (5 recycling cycles). Film samples were scissors cut into pieces (ca. 1 × 2 cm).

2.6.2. Characterization of virgin and multiple mechanically recycled PET films

Fourier Transform Infrared Spectroscopy (FTIR) was used to monitor chemical changes of extruded PET samples. Infrared spectra were obtained using a Perkin Elmer Spectrum One fitted with a universal attenuated total reflectance (ATR) sampling accessory (PerkinElmer, Waltham, USA), recorded over 16 scan cycles with a resolution of 4 cm^{-1} in the spectral range of 4000–650 cm^{-1} against air as background at room temperature (20 °C), at a resolution of 0.5 cm^{-1} under a fixed universal compression force of 80 N. FTIR results were used to determine the ester carbonyl index (CI) that is a parameter used to investigate the degree of degradation of PET samples before and after chemical and biological treatments, as expressed in the following Eq. (1):

$$CI = Band\ intensity\ at\ 1713\ cm^{-1}/Band\ intensity\ at\ 1408\ cm^{-1} \tag{1}$$

The thermal behavior of extruded PET samples was studied by Differential Scanning Calorimetry (DSC) recorded on a 2920 Modulated DSC (TA Instruments, New Castle, USA), previously calibrated with indium standard. Samples of 6 to 9 mg were weighted on an Explorer EX124 analytical balance (OHAUS Corporation, Parsippany, USA). Thermal analysis was conducted from 30 °C to 275 °C at a heating rate of 10 °C/min using nitrogen as purge gas at a flow rate of 30 mL/min. The crystallinity index (X_c) was calculated from the second heating cycle as follows (Eq. (2)):

$$X_c\ (\%) = ((\Delta H_m - \Delta H_c)/\Delta H_m^{\circ}) \times 100 \tag{2}$$

where, ΔH_m is the apparent melt enthalpy of the specimen tested, ΔH_c is the heat of cold crystallization, and ΔH_m° is a reference value that represents the heat of melting if the PET were 100% crystalline (140 J/g) (Wunderlich, 1973).

Scanning electron microscopy (SEM) images were obtained using Mira XMU SEM (Tescan™, Brno, Czech Republic) in backscattered electron mode for surface analysis. The accelerating voltage used was 10 kV. Prior to analysis, tested samples were placed on an aluminum stub and sputtered with a thin layer of gold using a Baltec SCD 005 sputter coater (New York, United States) for 110 s at 0.1 mbar vacuum.

2.6.3. Chemical recycling of virgin and recycled PET via microwave (MW) assisted hydrolysis

The efficiency of MW-assisted hydrolytic depolymerization of PET was evaluated following previously published work with slight modification (Azeem et al., 2022). Typically, V-PET, R₂-PET and R₅-PET films were separately mixed in 10% (w/v) sodium carbonate (Na$_2$CO$_3$) dissolved in 1 mL of EG. The sample suspensions were then MW irradiated at 350 W in a domestic microwave (MW) oven (Wavedom, LG, Seoul, South Korea) for 1.5 min. Dissolved PET was precipitated by the addition of distilled water. Finally, the obtained mixture was filtered, and the filtrate containing soluble monomers was analyzed by HPLC. The residual PET samples were dried overnight at 70 °C and kept in sealed bags for FTIR and DSC analysis. The depolymerization of PET was calculated using the following Eq. (3):

$$PET\ Conversion\ (\%) = (1 - \frac{Weight\ of\ residual\ PET}{Weight\ of\ initial\ PET}) \times 100 \tag{3}$$

The selectivity of soluble monomers was quantified from the HPLC chromatograms and the yield of TPA was calculated using Eq. (4):

$$Yield\ of\ TPA(\%) = \frac{(Conversion\ of\ PET\ (\%) \times Selectivity\ of\ TPA\ (\%))}{100} \tag{4}$$

The TPA monomer was then precipitated by the addition of 2 mL of concentrated HCl (34%, v/v) to the cooled filtrate. The separated TPA from each sample was washed with water, dried overnight at 70 °C and characterized by FTIR against TPA commercial standard.

2.7. Biodegradation of PET materials

2.7.1. B. subtilis BPM12 attachment to PET films

The attachment of *B. subtilis* BPM12 to PET films was assessed using the protocol reported by Ferrero et al. (2022). Briefly, an overnight culture of *B. subtilis* BPM12 (0.1%, v/v) was used to inoculate LB medium containing pieces of PET

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films (rinsed with EtOH (70%, *v/v*) and dried under laminar flow). After 7 days of incubation at 30 °C the films were rinsed with water and stained with crystal violet solution (1 g/L) for 20 min. The films were then destained using 30% (*v/v*) acetic acid and the measured absorbance (Epoch Microplate Spectrometer, BioTek, Winooski, USA) at 550 nm of the remaining solution was used as an indicator of cell attachment.

2.7.2. Biodegradation of PET films using whole cells

PET biodegradation experiments were performed in flasks with 25 mL of MSM medium containing glucose (20 g/L) as a carbon source. PET strips (cut into pieces of approximately 0.5 cm × 2.5 cm, rinsed with 70% (*v/v*) EtOH, dried under laminar flow and weighed) were added to flasks. The flasks were then inoculated with 1% (*v/v*) overnight culture of *B. subtilis* BPM12 (grown in MSM medium) and incubated at 30 °C, 180 rpm (MaxQ 6000, Thermo Fisher Scientific, Waltham, USA). Appropriate controls, without the addition of bacterial inoculum were also included. After 4 and 8 weeks, PET strips were taken out, washed with EtOH (70%, *v/v*), air dried and weighed and the medium supernatant was analyzed via HPLC.

2.7.3. Biodegradation of PET films using total protein preparations

Strain BPM12 was grown in LB supplemented with BHET (2 g/L) at 30 °C, 180 rpm until OD_{600} reached 5–6. The culture was centrifuged for 10 min at 5000 × *g* (Eppendorf centrifuge 5804, Hamburg, Germany) and the supernatant was stored at 4 °C until use. The cell pellet was resuspended in sodium phosphate buffer (20 mM, pH 7.2) supplemented with lysozyme, and incubated for 30 min at 37 °C, followed by sonication of 4 pulses of 15 s at 20 kHz (Soniprep 150, MSE (UK) Ltd., London, UK). The suspension was clarified by centrifugation for 30 min at 20000 × *g*, 4 °C (Eppendorf Centrifuge 5417 R, Hamburg, Germany) to obtain the cell-free extract. The total protein mixture was prepared by mixing cell-free extract and culture supernatant in equal volumes. The protein concentration of samples was determined using Bicinchoninic Acid Kit for Protein Determination (Sigma Aldrich, Hamburg, Germany) and adjusted to 500 µg/mL. Total protein preparations were stored at −20 °C until use.

Enzymatic biodegradation of recycled PET plastic films was performed in sodium phosphate buffer (20 mM, pH 7.2) using total protein mixture from strain BPM12. The experiments were performed in glass flasks, in 7 mL buffer volume, at 30 °C, 180 rpm, for 4 and 8 weeks. Aliquots (1 mL) of total protein preparations were added every week, while aliquots (1 mL) of tested samples were taken and stored at −20 °C for further HPLC analysis. The same procedure was applied to controls — PET plastic films in sodium phosphate buffer, which was also exchanged weekly.

After biodegradation experiments, PET plastic films were washed with EtOH (70%, *v/v*), air dried and weighed. All samples were analyzed via SEM analysis as previously described for characterization of virgin and multiple mechanically recycled PET films.

2.8. Statistical analysis

The results are presented as mean ± standard deviation (SD). Statistical analysis was done by comparing means using *t*-test (Two-Sample Assuming Equal Variances) and one-way ANalysis Of VAriance (ANOVA, Single Factor), with Fisher's Least Significant Difference (LSD) post-hoc test. The level of statistical significance is expressed as a *p*-value (probability value), and p ≤ 0.05 was considered statistically significant. Statistical analysis tests were performed in Microsoft Excel Spreadsheet Software by Data Analysis Tools add-in.

3. Results and discussion

3.1. Isolation and identification of Bacillus sp. BPM12

BPM12 is a mesophilic bacterium isolated from the pristine soil sample from the mountain slope with limited vegetation. During the phenotypic screening, it was distinguished by its ability to efficiently grow on BHET, PCL and Impranil DL 2077 as a sole carbon sources, using MSM medium. Clearing halos on BHET plates were observed after three days of incubation at 30 °C, suggesting BPM12 a potentially useful strain for PET and other plastics degradation (Fig. 1a). The strain could grow at temperatures from 20 to 47 °C and at pH values from 6 to 10. It fermented 26 out of the 49 carbohydrates, including simple sugars such as mannose, fructose, and glucose but also polysaccharides such as starch and glycogen (Table S2). It was not able to grow on TPA as the sole carbon source. The 16S rRNA gene sequence placed BPM12 within the *Bacillus* genus most closely related to *B. subtilis* strain BAB-1684 (Accession number: KF535143.1) with 99% sequence identity (Fig. 1b). The strain was named *Bacillus* sp. BPM12 and the 16S sequence was deposited to GenBank under the accession number OQ381249.

BPM12 grew equally well on minimal and nutrient rich solid media within 24 h of inoculation. It formed creamy-white and orange colonies with smooth irregular edges on MSM and MSF, respectively. Colonies on LB plates were opaque and circular. The growth of *Bacillus* sp. BPM12 was also compared to *B. subtilis* 168 on different media (Fig. S3a). Neither strain exhibited hemolytic activity. Fluorescent microscopy revealed BPM12 cells were rod-shaped with an approximate size of 0.8–1.0 × 5–7 µm which is consistent with *Bacillus* spp. morphology (Fig. S3b).

Bacillus is a remarkably diverse bacterial genera, able to grow within ecologically diverse environments (Earl et al., 2008). *Bacillus* strains have been investigated for xenobiotic degradation such as the degradation of pesticides cypermethrin, imidacloprid, fipronil, and sulfosulfuron reaching degradation rates of up to 99% (Gangola et al., 2021, 2022).
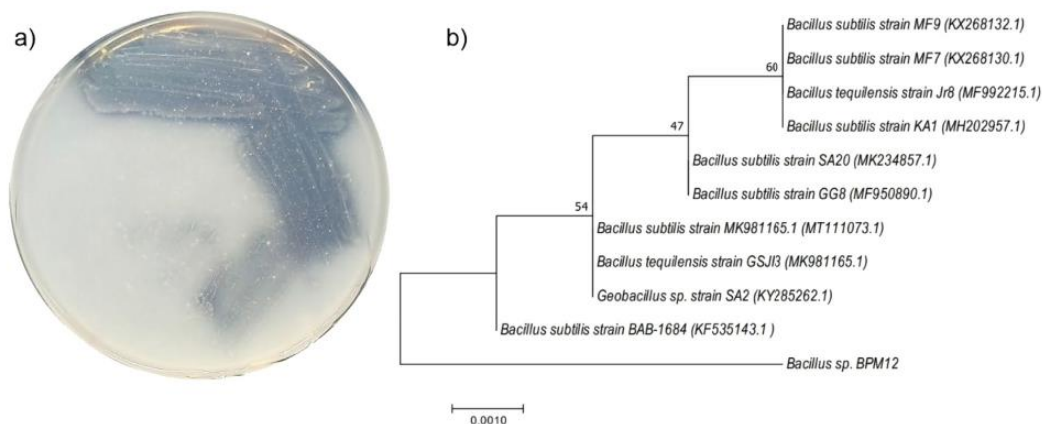
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Fig. 1. (a) *Bacillus* sp. BPM12 forming clearing zones on MSM agar plate with BHET as the sole source of carbon and energy after 3 days at 30 °C; (b) Maximum likelihood tree showing the relationship of *Bacillus* sp. BPM12 to 10 of the most closely related strains. Bootstrap values based on 1000 replications are displayed on the nodes of the tree. The scale bar represents genetic distance.

Members of the *Bacillus* genus have been reported to degrade various plastic polymers including PET (Ribitsch et al., 2011), polyurethanes (Shah et al., 2013) and polylactic acid (Bonifer et al., 2019) and have been identified in several consortiums capable of degrading recalcitrant plastics (Roberts et al., 2020; Shah et al., 2016; Skariyachan et al., 2017). *Bacillus* sp. BPM12 growth profile at temperatures above 40 °C as well as tolerance towards alkaline conditions matches that of some previously reported *Bacillus* strains (Ali et al., 2016; Hanim, 2017; Wang et al., 2019) and is highly desirable for biotechnological applications where biocatalysts need to withstand harsh conditions. Another valuable trait of *B. subtilis* is the ability to form highly resistant endospores in response to nutrient deprivation and other environmental stresses, which had already been used for efficient surface display of relevant enzymes including PETases (Jia et al., 2022). Therefore, *Bacillus* sp. BPM12 was further investigated as a potential biocatalyst for PET degradation.

3.2. Degradation of PET-related model substrates

Resting whole cells of *Bacillus* sp. BPM12 were able to hydrolyze BHET, 1MER and 2MER, while 1.5MER and 3MER showed only traces of degradation products, based on HPLC and TLC analysis (Fig. 2; Figs. S4–S6). During the course of the reaction, 1000 mg/L of BHET was completely converted to MHET and TPA within 120 h at 30 °C (Fig. 2a). The ratio of TPA to MHET was 1:6 within this time period. The control reactions showed some BHET auto-hydrolysis to MHET ($\leq$ 5%, w/w). These results suggest that BHET is firstly converted to MHET that is subsequently converted to TPA at a considerably slower rate, a trend also observed among different PET degrading enzymes (Mrigwani et al., 2022). *Bacillus* sp. BPM12 is more efficient in BHET conversion when compared to *Enterobacter* sp. HY1, which was able to degrade 80.8% of BHET (1000 mg/L) in 120 h at 30 °C (Qiu et al., 2020), and comparable to a *Yarrowia lipolytica* wild-type (Wt) strain which could convert 500 mg/L of BHET in about 48 h at 29 °C (da Costa et al., 2020). However, engineered strains expressing IsPETase achieve much higher conversion rates, reaching up to 5 g/L and 2 g/L when the enzyme is expressed in *Y. lipolytica* Po1fP and in *B. subtilis*, respectively (Qi et al., 2021).

1MER of PET was also found to be fully converted to mono-methyl terephthalate (MTPA), MHET and TPA, suggesting that *Bacillus* sp. BPM12 can cleave both ester bonds of 1MER (Fig. 2b). Given that the main product detected was MTPA, when 1MER was used as a substrate, the preferred cleavage site was at the ethyl moiety. The slow conversion of MHET and MTPA to TPA by *Bacillus* sp. BPM12 may be due to MHET inhibition as it has been shown that MHET considerably inhibited the hydrolytic activity of Bs2Est (Kim et al., 2021). 1.5MER and 2MER were converted to MTPA, MHET and TPA confirming both exo- and endo-cleaving activity of *Bacillus* sp. BPM12 (Figs. S4 and S5). 2MER is most likely firstly converted to MTPA and BHET via endo-cleaving activity and then broken down to MHET and TPA via exo-cleaving activity, a mechanism previously shown when Bs2Est was used as a biocatalyst (Kim et al., 2021). 3MER was found to be much harder to degrade with only traces of degradation products detected, which can be contributed to the poor solubility and high hydrophobicity of this substrate (Fig. S6). Similarly, recently described polyesterase from *Moraxella* sp. (MoPE) capable of degrading highly crystalline PET was characterized using the same set of substrates revealing the same mode of action (Nikolaivits et al., 2022).
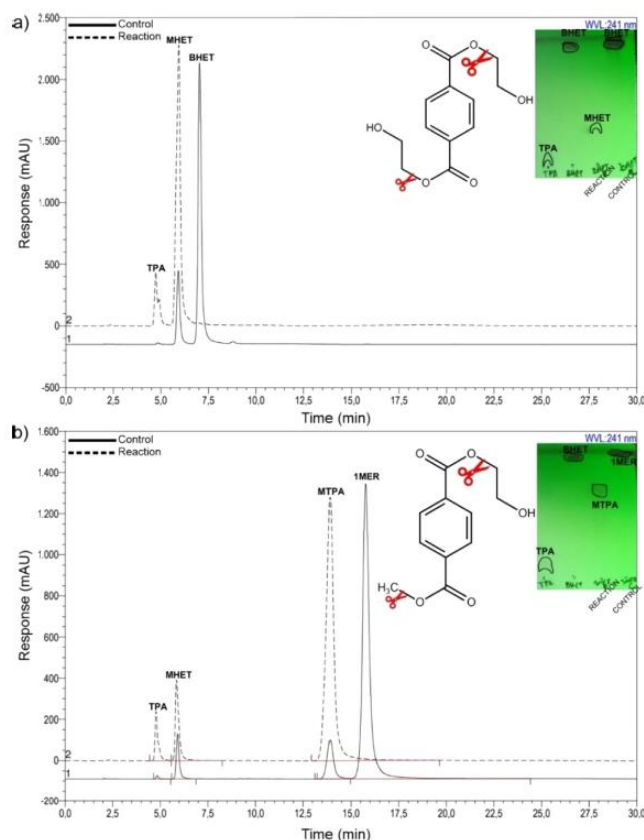
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Fig. 2. BHET and 1MER biotransformation using whole cells of *Bacillus* sp. BPM12 was monitored via HPLC and TLC. (a) BPM12 resting cells transformation of BHET; (b) BPM12 resting cells transformation of 1MER of PET. In the HPLC chromatograms, the full lines represent the control reactions (no biocatalyst), while the dashed lines represent the reactions containing BPM12 cells.

3.3. Bacillus sp. BPM12 genome analysis

The genome assembly consisted of 137 contigs, is 4160070 bp long and is 98.6% complete based on BUSCO (Benchmarking Universal Single-Copy Orthologs) analysis. Strain BPM12 was confirmed to be *B. subtilis* sp. with the Genome Taxonomy Database Toolkit. The predicted proteome of BPM12 consists of 4196 proteins and 3406 of them were clustered in 3301 homologous groups with proteins from the other three *Bacillus* strains with reported activity on PET polymer. Almost half of the total BPM12 proteins are core proteins with homologs in all considered genomes and most BPM12 proteins were clustered with proteins of the AIIW2 strain with which it shares 78.2% whole-genome average nucleotide identity (Fig. 3).

Bacillus sp. AIIW2 is a marine isolate that was found to utilize PET as a carbon source (Kumari et al., 2021). *B. thuringiensis* C15 and *B. albus* PFYN01 are members of a 5 strain consortium that grew synergistically in the presence of PET as the sole carbon source (Roberts et al., 2020). Both strains tested negative for lipase activity with C15 being unable to grow on PET in the absence of the rest of the consortium.

The proteome of BPM12 contains 5 enzymes that share high similarity with known plastic-active enzymes from PAZy (Table 1). These 5 enzymes include three esterases and two serine proteases. The two serine proteases are core genes with homologs in all considered genomes. The CE WP_216995529.1 is almost 100% identical to the intracellular PETase from *B. subtilis* strain 4P3-11 that can hydrolyze 3PET and PET films (Ribitsch et al., 2011) and has no homologs in the other three proteomes. No significant homology was detected with known MHET-ases such as Mle046 (Meyer-Cifuentes and Öztürk, 2021). The lipase LipA and the esterase EstB are both secreted and are members of the same InterPro family (IPR002918). They form a homologous cluster with an alpha/beta hydrolase from isolate AIIW2 that lacks a signal peptide. Kumari et al. report a non-secreted CE (accession number: WP_020451834.1) displaying the highest relative fold change in the
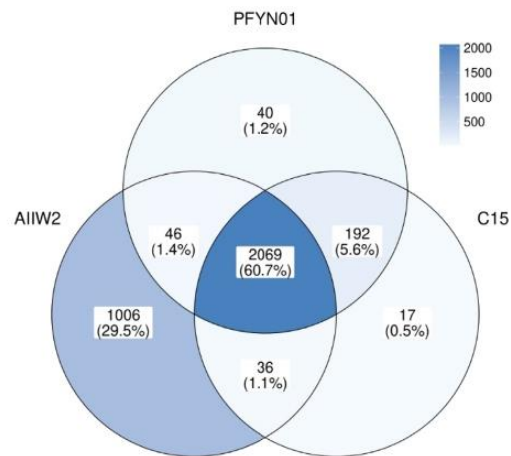
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Fig. 3. *B. subtilis* BPM12 proteins clustered in homologous groups with proteins from known PET-active *Bacillus* strains (*B. albus* PFYN01, *Bacillus* sp. AllW2, and *B. thuringiensis* C15).

Table 1
B. subtilis BPM12 proteins and their PAZy homologs.

BPM12	PAZy	Identity %	Active on
Carboxylesterase (WP_216995529.1)	PETase (ADH43200.1)	98.9	PET
Subtilisin AprE (WP_015715621.1)	*Subtilisin carlsberg* (P00780)	65.2	PLA
Lipase LipA (WP_086343408.1)	PLaA (Q83VD0)	48.8	PLA
Esterase EstB (WP_003243184.1)	PLaA (Q83VD0)	46.1	PLA
Serine protease Isp (WP_029946400.1)	Subtilisin savinase (P29599.1)	45.4	PLA

presence of PET based on quantitative reverse transcriptase–polymerase chain reaction analysis (Kumari et al., 2021). This CE clustered with the alpha/beta hydrolase WP_014480039.1 of BPM12. The two enzymes share 68% sequence identity and have the same length.

The genome of BPM12 was further searched for enzymes with esterase activity, the main activity associated with PET degradation but involved in pesticide degradation as well (Gangola et al., 2018), based on the functional protein domains identified with InterProScan. This analysis identified 63 esterases with 7 of them being predicted to be secreted. Only one of the secreted esterases, the phosphodiesterase WP_264240018.1, is a core gene present in all considered genomes. The GDSL esterase WP_264240216.1 has no homologs in any other isolate and the remaining 5 secreted esterases, including LipA and estB, have homologs only in the AllW2 genome. Recently, pangenomic analysis that included 88 *Bacillus* species, revealed many other biodegradation genes involved in plastics and plasticizers degradation through the Plastic Microbial Biodegradation Database (PMBD) apart from the genes implicated in PET degradation (Edwards et al., 2022). An esterase from *B. subtilis* was immobilized on halloysite nanotubes and completely degraded dibutyl phthalate (Balci et al., 2023). Furthermore, *Bacillus* species were also shown to produce a number of valuable proteases and enzymes involved in metal tolerance and removal (Liya et al., 2023; Sharma et al., 2022).

3.4. Expression of BPM12CE

Given the high sequence identity of BPM12CE (Table S3) to a known PETase from *B. subtilis* (WP_216995529.1), BPM12CE was selected as the most likely gene responsible for the PET-related substrate degradation activity. Therefore, the gene was deleted from the genome of *B. subtilis* BPM12 to create a knockout mutant. The deletion of Bpm12CE indeed led to the almost complete loss of BHET degrading activity (Fig. S7a) with only a faint clearing halo visible, as well as the loss of ability to grow on BHET as the sole carbon source, corroborating the hypothesis that *B. subtilis* BPM12 utilizes EG during growth on BHET.

To further investigate its BHET degrading activity *bpm12CE* gene was cloned into a *Bacillus* expression vector and introduced into both *B. subtilis* BPM12 and 168. The general esterase activity using pNPB was detected in both strains and in both intracellular and extracellular protein fractions (Fig. S8). The relative intracellular esterase activity was generally lower in comparison to extracellular protein fractions (between 1.4- and 5.9-fold) but remained comparable between the strains. Nevertheless, the extracellular esterase activity of recombinant strains increased by 1.8- and 3.2- fold in *B. subtilis* BPM12 and *B. subtilis* 168, respectively, compared to Wt strains. To get a better insight into specific BHETase
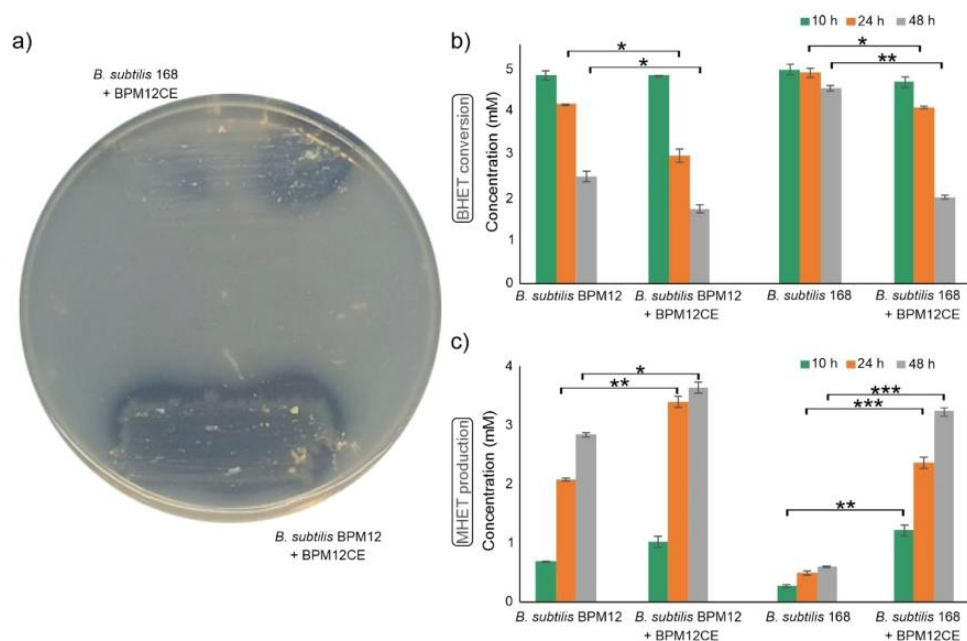
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Fig. 4. Growth and activity of BPM12 Wt and B. subtilis 168 strains expressing BPM12CE. (a) MSM agar plates with BHET as the sole carbon source after 10 h, 24 h and 48 h at 30 °C; (b) BHET conversion in liquid culture; (c) MHET production in liquid culture after 10 h, 24 h and 48 h incubation. Results were analyzed using ANOVA test and post-hoc Fisher's LSD test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

activity, the recombinant strains were tested using BHET as a substrate on plates and in liquid biotransformation reactions using whole cells (Fig. 4). Initially, *B. subtilis* 168 did not form clearing halos on BHET-containing plates while BPM12 did (Fig. 1, Fig. S7b). When transformed, both strains exhibited BHET degrading activity, however, recombinant *B. subtilis* BPM12 was able to grow better and form larger zones of clearance on MSM agar containing BHET as a sole carbon source (Fig. 4a). In liquid culture, BHET degradation by *B. subtilis* 168 harboring BPM12CE showed a 6-fold increase in comparison to untransformed *B. subtilis* 168 (Fig. 4b), clearly demonstrating that BPM12CE was indeed responsible for BHET conversion. Furthermore, the activity of recombinant *B. subtilis* 168 was almost identical to that of *B. subtilis* BPM12 Wt strain. Homologous expression of BPM12CE in *B. subtilis* BPM12 led to 1.6- fold increase in the MHET production within the first 24 h in comparison to the Wt strain (Fig. 4c).

Bacillus is a more favorable expression system in comparison to *E. coli* due to intrinsic secretion capacity and better protein folding resulting in a higher yield of more stable enzymes (Souza et al., 2021; Wei et al., 2019). Four enzymes with PETase activity have been successfully expressed in *Bacillus* species so far. *T. fusca* hydrolase (TfH) was obtained using *B. megaterium* with an excellent yield of 240 μg/L at a 2 L scale (Yang et al., 2007). Subsequently, a highly similar enzyme TfCut2 was also obtained from *B. subtilis* with higher purity and its activity on PET materials was demonstrated. It was shown that TfCut2 is more thermostable when expressed in *Bacillus* compared to *E. coli* with a 4 °C higher melting point (Wei et al., 2019). Signal peptide optimization allowed for the enhanced secretion of IsPETase (Huang et al., 2018; Wang et al., 2020) and BhrPETase (Xi et al., 2021). Given that *B. subtilis* BPM12 possesses intrinsic activity towards PET-related substrates it could serve as an ideal platform for the expression of various PETases and other auxiliary enzymes to increase polymer degrading capacity.

3.5. Preparation, characterization and chemical recycling of PET materials

Mechanical recycling has been the most common method used to recover PET and other recyclable plastics because it is relatively easy and economical (Faraca et al., 2019). Nevertheless, the cleavage of the long polymer chains caused by thermomechanical degradation is a common problem that affects the properties of mechanically recycled PET during reprocessing and lifetime (Makkam and Harnnarongchai, 2014). The resulting shorter polymer chains are expected to be more susceptible to biodegradation, which succeeds abiotic degradation (Mohanan et al., 2020).

The properties of the virgin and recycled PET materials obtained from the chemical and thermal analysis are shown in Table 2. Chemical analysis by FTIR accessed possible chemical changes due to thermomechanical degradation of PET chains

Table 2
Properties of the untreated virgin and recycled PET films and their respective residues obtained from post MW-assisted depolymerization treatment.

Sample	Untreated			MW-assisted treatment		
	T_m (°C)[a]	X_c[b]	CI[c]	T_m (°C)[a]	X_c[b]	CI[c]
V-PET	246.1	34.9	4.8	246.8	19.5	0.6
R_2-PET	249.0	38.6	4.4	232.3	24.8	3.2
R_5-PET	250.2	44.5	4.2	220.9	11.1	1.4

[a]Melting temperature;
[b]Crystallinity index;
[c]Carbonyl index.

over the reprocessing cycles (Holland and Hay, 2002). The IR spectra of virgin and recycled PET samples are shown in the 1900–650 cm^{-1} range (Fig. S9). The band at 1570 cm^{-1} is related to a conjugated aromatic structure in the PET samples, while the region from 950 to 750 cm^{-1} is attributed to C–H deformation. On one hand, no significant changes in the aforementioned bands were observed for both R_2-PET and R_5-PET in comparison to the IR spectrum of V-PET, suggesting no clear sign of thermal degradation resulting from the extrusion process. Accordingly, it can be seen from Table 2 that the mechanical recycling process had no significant impact on the *CI* for the studied wavenumber that rather decreased slightly from 4.84 in the V-PET to 4.42 and 4.18 in the R_2-PET and R_5-PET materials, respectively. On the other hand, there were clear changes in the intensity of the bands at 1470, 1370 and 1340 cm^{-1} assigned to CH_2 bending and wagging modes of trans conformers, which have been associated with the degree of crystallinity of the PET materials (Sammon et al., 2000). This is evidenced by a subsequent increase in both the glass transition temperature (T_g) and crystallinity index (X_c) calculated from the second heating step of the DSC thermograms of reprocessed R_2-PET and R_5-PET materials when compared to the values obtained for V-PET (Fig. S10). In particular, the role of the crystalline phase in the performance of recycled PET has been investigated elsewhere (Badia et al., 2012). Therefore, the increase of the X_c observed herein ranging from 34.99 in the V-PET to 44.48 in the R_5-PET material was attributed to the growth of more crystalline domains promoted by the formation of shorter polymer fragments that resulted from the cleavage of the polymer backbone, and possibly act as nuclei upon crystallization.

V-PET, R_2-PET and R_5-PET underwent chemical recycling via MW-assisted hydrolysis. The residual PET obtained was weighed and the conversion of PET (%) was calculated. The reaction products dissolved in the cooled filtrate were analyzed by HPLC. The effects of different PET pretreatment on the conversion of PET, the selectivity of soluble monomers and the yield of TPA are illustrated in Fig. S11. It was observed that the rate of conversion was only 75.4% when applying MW-assisted hydrolysis reaction on untreated virgin PET and then increased to 86.9 and 94.2% when hydrolyzing R_2-PET and R_5-PET, respectively. Such a significant increase in depolymerization efficiency with the mechanically recycled PET at only 1.5 min MW irradiation time could be attributed to the modifications that took place in the properties of the virgin PET after several cycles of mechanical recycling. The yield of TPA increased from 36.5% for the virgin PET sample to 38.1% and 56.1% for R_2-PET and R_5-PET, respectively (Fig. S11). Noticeably, the selectivity of MHET was much higher than that of BHET for all samples. It was also observed that the selectivity of BHET and MHET were almost the same for all chemically treated samples indicating that modifications that occurred by the pretreatment process did not have a significant effect on the selectivity of depolymerization products obtained post chemical recycling. Moreover, the residual PET obtained post chemical recycling process for the studied PET samples showed a decrease in the crystallinity and carbonyl index from the original samples as demonstrated in Table 2. The X_c of the obtained residues ranged between 11 and 25 while the carbonyl index ranged between 0.6 and 3.2. It is also worth mentioning that the DSC results of the obtained residues post chemical recycling process, especially R_2-PET and R_5-PET, have shown a decrease in the melting point temperatures in comparison to the original samples. This could be attributed to the production of lower molecular weight PET oligomers after the chemical recycling process exhibiting a melting endotherm peaking at temperatures lower than that of untreated PET (Chaudhary et al., 2013). The identity of the TPA white powder monomer precipitated post the MW-assisted hydrolysis process was confirmed using FTIR analysis (Fig. S12). FTIR spectra of precipitated TPA from all treated PET samples were almost identical to standard TPA and TPA reported in the literature (Azeem et al., 2022).

3.6. Degradation of PET material samples by B. subtilis BPM12 whole-cells and total protein extracts

As previously reported, biofilm formation is an important factor contributing to the initiation of plastic degradation (Maheswaran et al., 2023), therefore the ability of *B. subtilis* BPM12 to attach to PET films was assessed. *B. subtilis* BPM12 showed the ability to form biofilms on both, virgin and recycled PET films (Fig. 5a).

The recycling process apparently increased the ability of cell attachment to the films, possibly due to changes on the surface of the films. However, whole-cell degradation of PET materials using *B. subtilis* BPM12 did not lead to any detectable weight changes after 8 weeks of incubation, while weight changes after enzymatic biodegradation of virgin and recycled PET films are represented in Table S4. Although the weight loss of PET materials was minimal and comparable amongst samples, HPLC analysis revealed the release of PET degradation products, mostly TPA and MHET when cell-free
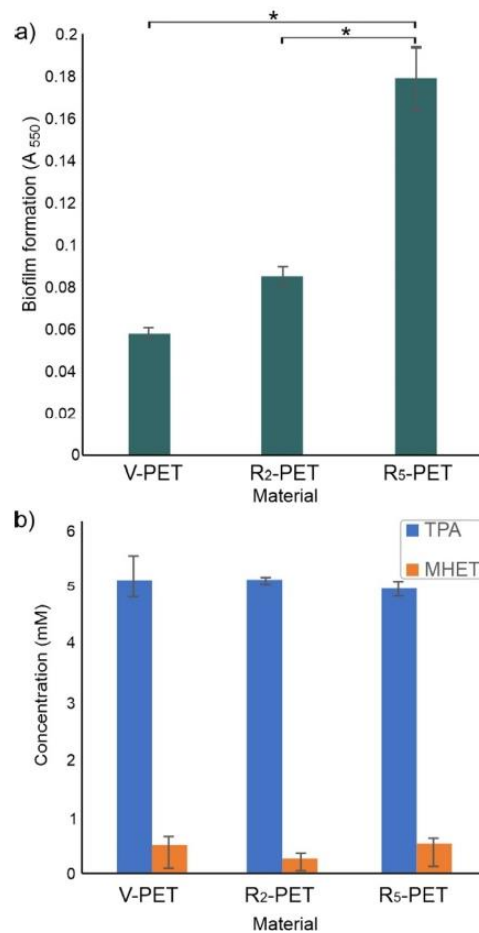
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Fig. 5. *B. subtilis* BPM12 attachment on PET materials (a) and biodegradation products (TPA and MHET) detected after 8 weeks (b) of incubation of PET materials with total protein preparation at 30 °C. Results were analyzed using ANOVA test and post-hoc Fisher's LSD test, $p \leq 0.05$ was considered statistically significant.

enzymes from this strain were incubated with materials over this time period (Fig. 5b). Although the initial attachment of the whole cells was 2-fold higher in the case of re-extruded materials, the yield of degradation products using enzymes from this strain was comparable for all used materials. As in the case of PET MW-assisted hydrolysis, material properties after enzymatic treatment were assessed (Table 3). Indeed, enzymatic treatment did not have a significant effect on the materials' T_m or *CI*. However, the X_c for R$_5$-PET has increased by 40% in comparison to that of the untreated R$_5$-PET material (Table 2), which further implies that enzymatic activity was focused on amorphous regions of the material.

These results were further confirmed by SEM analysis of non-treated and enzymatically treated materials. As presented in Fig. 6, the surface of non-treated virgin PET is smooth, with few abrasions, while in the case of recycled (re-extruded) PET, multiple surface plications were detected, which explains biofilm formation susceptibility of these samples (Perera-Costa et al., 2014). Furthermore, a clear difference between treated and control samples has been observed. Surface modifications in the form of cracks and dents could be a result of degradation of the preferred amorphous regions in the materials while crystalline structures have remained intact.

The preference of PET-degrading enzymes towards amorphous regions has been discussed in various studies (Kawai et al., 2019; Tournier et al., 2020), however further analysis is required in order to determine the exact fractions composition of the samples (rigid amorphous fraction, mobile amorphous fraction, crystallinity degree). Changes on the surface of examined materials imply that enzymatic treatment has led to materials surface erosion which is in accordance with the biodegradation product release detected (Fig. 5b).

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Table 3
Properties of the virgin and recycled PET residues obtained post BPM12 enzymatic treatment.

Sample	T_m (°C)[a]	X_c[b]	CI[c]
V-PET	246.1	34.9	4.9
R_2-PET	249.0	38.6	4.3
R_5-PET	250.0	62.3	4.2

[a]Melting temperature;
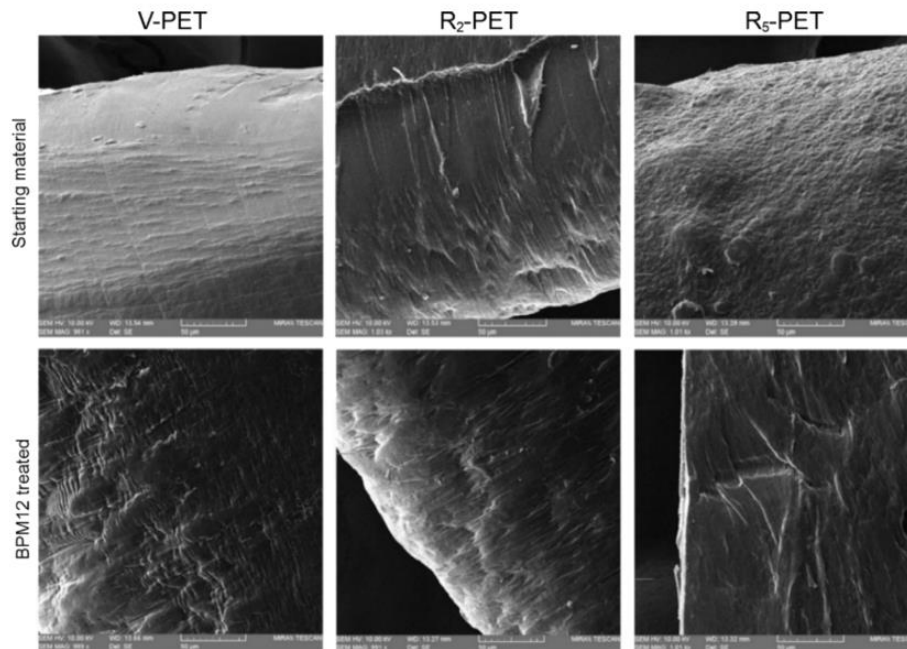[b]Crystallinity index;
[c]Carbonyl index.

Fig. 6. SEM images of V-PET, R_2-PET, and R_5-PET films before and after the exposure to BPM12 biodegradation for 8 weeks (1000 × magnification, scale bar = 50 μm).

Additionally, erosion degree appears to correlate with BHET and MHET release. Similar surface modifications have been previously reported by Chen et al. in a study where the whole-cell biocatalyst was engineered to improve degradation of highly crystalline PET materials (crystallinity over 45%) (Chen et al., 2022). After the exposure of materials to BPM12, virgin PET (sample with the lowest crystallinity percentage) has shown the most significant surface modification correspondingly with the study by Thomsen et al. where it was shown that PET degradation is directly correlated to crystallinity percentage (Thomsen et al., 2022).

Taken together, polymeric PET both mechanically recycled and virgin, is not a suitable substrate for microbial or enzymatic attack for this strain. However, enzymatic treatment would be suitable as follow up treatment of mild hydrolysis, where there is oligomeric byproduct of considerable amount generated (25% in the case of virgin PET material; Fig. S11). This substrate would be suitable for biocatalytic depolymerization, as it was shown that crude enzyme preparations from strains that can use BHET as sole source of carbon and energy can efficiently depolymerase PET oligomers (Fig. 2; Figs. S4–S6).

4. Practical applications and future research prospects

Keeping in mind the high volume of newly produced PET materials, it is of high importance to develop mixed mechano/bio-degradation processes to achieve decreased carbon footprint and increase the scope of PET recycling beyond high-grade high-purity PET waste streams. Further research should be directed towards biocatalyst improvement through

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enzyme engineering and other optimizations of the biocatalytic process. Namely, the possibility to drive the degradation to completeness and efficiently recover EG and TPA produced should be further explored. The research should also be extended with other types of PET materials, including mixed and postconsumer ones.

5. Conclusions

A new *B. subtilis* BPM12 strain with high BHET degradation activity and the ability to attach and form biofilms on PET films was described. A novel carboxylesterase, highly homologous to a previously described intracellular PETase was identified through genome sequencing and overexpressed in both *B. subtilis* BPM12 and 168, resulting in increased BHET-ase activity. It was also demonstrated that *B. subtilis* BPM12 could serve as an ideal platform for the expression of PETases and other auxiliary enzymes to increase polymer degrading capacity. In pursuit of a combined mechano/bio-degradation approach, mixed mechanical and green chemical hydrolysis recycling was carried out resulting in by-products containing PET oligomers and other partially degraded products. This treatment resulted in substrates that are much more suited for biocatalytic treatment and has the potential to be an important step in achieving more favorable and sustainable routes to advance plastic waste circularity and upcycling.

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CRediT authorship contribution statement

Brana Pantelic: Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing. **Jeovan A. Araujo:** Methodology, Investigation, Writing – original draft. **Sanja Jeremic:** Methodology, Investigation, Writing – original draft. **Muhammad Azeem:** Methodology, Investigation, Writing – original draft. **Olivia A. Attallah:** Methodology, Investigation, Writing – original draft, Visualization. **Romanos Siaperas:** Investigation, Methodology, Writing – original draft. **Marija Mojicevic:** Conceptualization, Validation, Writing – review & editing. **Yuanyuan Chen:** Investigation, Methodology, Writing – original draft. **Margaret Brennan Fournet:** Conceptualization, Validation, Writing – review & editing. **Evangelos Topakas:** Methodology, Resources, Investigation, Writing – review & editing, Supervision. **Jasmina Nikodinovic-Runic:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

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 paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2023.103316.PET-related model substrates used to investigate the biocatalytic potential of B. subtilis BPM12 (Fig. S1); Illustration of the mechanical recycling process using a twin-screw extruder for the fabrication and reprocessing of PET films (Fig. S2). Growth of *B. subtilis* BPM12 compared to *B. subtilis* 168 on LA, MSF, and MSM and blood agar plates (a) and fluorescent microscopy of *B. subtilis* BPM12 under 100 000 x magnification (b). 1.5MER, 2MER and 3MER biotransformation using whole cells of *B. subtilis* BPM12 was monitored via HPLC and TLC (Fig. S4-S6). Growth of *B. subtilis* BPM12 and the knockout mutant *B. subtilis* BPM12 $\Delta bpm12CE$ on MSM plates containing BHET as the sole carbon source b) Growth of *B. subtilis* 168 on MSM plates containing BHET as the sole carbon source; c) Growth of recombinant *Bacillus* strains transformed with BPM12CE on MSM plates containing BHET as the sole carbon source 5 g/L (Fig. S7). General esterase activity of intracellular and extracellular enzyme fractions of recombinant and Wt *B. subtilis* BPM12 and *B. subtilis* 168 (all values are standardized based on protein concentration) (Fig. S8). FTIR spectra of the V-PET, and the reprocessed R_2-PET and R_5-PET materials are shown in the wavenumber range 1900–650 cm^{-1} (Fig. S9). DSC thermograms of the second heating step of V-PET, R_2-PET, and R_5-PET from 30 °C to 275 °C (10 °C/min) under nitrogen atmosphere (30 mL/min) (Fig. S10). The effect of re-extrusion process on the conversion of PET to BHET, MHET and the yield of TPA via MW-assisted hydrolysis using Na_2CO_3 in ethylene glycol (Fig. S11). FTIR spectra of [a] TPA standard, [b] TPA obtained from V-PET depolymerization, [c] TPA obtained from R_2-PET depolymerization and [d] TPA obtained from R_5-PET depolymerization (Fig. S12). Primers used for *bpm12CE* cloning and construction of the knockout mutant *B. subtilis* BPM12 $\Delta bpm12CE$ (Table S1). The ability of *B. subtilis* BPM12 to metabolize different carbohydrates (Table S2). Nucleotide and amino acid sequence of BPM12CE (Table S3). Weight changes after enzymatic degradation of PET films (Table S4).

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References

Ali, N., Ullah, N., Qasim, M., Rahman, H., Khan, S.N., Sadiq, A., Adnan, M., 2016. Molecular characterization and growth optimization of halo-tolerant protease producing *Bacillus subtilis* strain blk-1.5 isolated from salt mines of Karak, Pakistan. Extremophiles 20, 395–402. http://dx.doi.org/10.1007/s00792-016-0830-1.

Azeem, M., Fournet, M.B., Attallah, O.A., 2022. Ultrafast 99% polyethylene terephthalate depolymerization into value added monomers using sequential glycolysis-hydrolysis under microwave irradiation. Arab. J. Chem. 15, 103903. http://dx.doi.org/10.1016/j.arabjc.2022.103903.

Badia, J.D., Strömberg, E., Karlsson, S., Ribes-Greus, A., 2012. The role of crystalline, mobile amorphous and rigid amorphous fractions in the performance of recycled poly (ethylene terephthalate) (PET). Polym. Degrad. Stab. 97, 98–107. http://dx.doi.org/10.1016/j.polymdegradstab.2011.10.008.

Balci, E., Rosales, E., Pazos, M., Sofuoglu, A., Sanromán, M.A., 2023. Immobilization of esterase from *Bacillus subtilis* on halloysite nanotubes and applications on dibutyl phthalate degradation. Environ. Technol. Innov. 30, 103113. http://dx.doi.org/10.1016/j.eti.2023.103113.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., 2012. Spades: A new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19, 455–477. http://dx.doi.org/10.1089/cmb.2012.0021.

Benyathiar, P., Kumar, P., Carpenter, G., Brace, J., Mishra, D.K., 2022. Polyethylene terephthalate (PET) bottle-to-bottle recycling for the beverage industry: A review. Polymers 14 (2366), http://dx.doi.org/10.3390/polym14122366.

Bonifer, K.S., Wen, X., Hasim, S., Phillips, E.K., Dunlap, R.N., Gann, E.R., DeBruyn, J.M., Reynolds, T.B., 2019. Bacillus pumilus B12 degrades polylactic acid and degradation is affected by changing nutrient conditions. Front. Microbiol. 10 (2548), http://dx.doi.org/10.3389/fmicb.2019.02548.

Buchholz, P., Zhang, H., Perez-Garcia, P., Nover, L.-L., Chow, J., Streit, W.R., Pleiss, J., 2021. Plastics degradation by hydrolytic enzymes: The plastics-active enzymes database-PAZy. Proteins 90, 1443–1456. http://dx.doi.org/10.1002/prot.26325.

Chaudhary, S., Surekha, P., Kumar, D., Rajagopal, C., Roy, P.K., 2013. Microwave assisted glycolysis of poly (ethylene terepthalate) for preparation of polyester polyols. J. Appl. Polym. Sci. 129, 2779–2788. http://dx.doi.org/10.1002/app.38970.

Chaumeil, P.-A., Mussig, A.J., Hugenholtz, P., Parks, D.H., 2020. Gtdb-tk: A toolkit to classify genomes with the genome taxonomy database. Oxf. Univ. Press 36, 1925–1927. http://dx.doi.org/10.1093/bioinformatics/btz848.

Chen, Z., Duan, R., Xiao, Y., Wei, Y., Zhang, H., Sun, X., Wang, S., Cheng, Y., Wang, X., Tong, S., Yao, Y., Zhu, C., Yang, H., Wang, Y., Wang, Z., 2022. Biodegradation of highly crystallized poly(ethylene terephthalate) through cell surface codisplay of bacterial petase and hydrophobin. Nature Commun. 13, 7138. http://dx.doi.org/10.1038/s41467-022-34908-z.

Commission, E., 2022. Commission regulation (eu) 2022/1616 of 15 september 2022 on recycled plastic materials and articles intended to come into contact with foods. https://eur-lex.europa.eu/eli/reg/2022/1616/oj.

Contreras-Moreira, B., Vinuesa, P., 2013. Get_homologues, a versatile software package for scalable and robust microbial pangenome analysis. Appl. Environ. Microbiol. 79, 7696–7701. http://dx.doi.org/10.1128/AEM.02411-13.

da Costa, A.M., de Oliveira Lopes, V.R., Vidal, L., Nicaud, J.-M., de Castro, A.M., Coelho, M.A.Z., 2020. Poly (ethylene terephthalate)(PET) degradation by *Yarrowia lipolytica*: Investigations on cell growth, enzyme production and monomers consumption. Process Biochem. 95, 81–90. http://dx.doi.org/10.1016/j.procbio.2020.04.001.

Diao, J., Hu, Y., Tian, Y., Carr, R., Moon, T.S., 2023. Upcycling of poly (ethylene terephthalate) to produce high-value bio-products. Cell Rep. 42, 111908. http://dx.doi.org/10.1016/j.celrep.2022.111908.

Djapovic, M., Milivojevic, D., Ilic-Tomic, T., Lješević, M., Nikolaivits, E., Topakas, E., Maslak, V., Nikodinovic-Runic, J., 2021. Synthesis and characterization of polyethylene terephthalate (PET) precursors and potential degradation products: Toxicity study and application in discovery of novel petases. Chemosphere 275, 130005. http://dx.doi.org/10.1016/j.chemosphere.2021.130005.

Earl, A.M., Losick, R., Kolter, R., 2008. Ecology and genomics of *Bacillus subtilis*. Trends Microbiol. 16, 269–275. http://dx.doi.org/10.1016/j.tim.2008.03.004.

Edwards, S., León-Zayas, R., Ditter, R., Laster, H., Sheehan, G., Anderson, O., Beattie, T., Mellies, J.L., 2022. Microbial consortia and mixed plastic waste: Pangenomic analysis reveals potential for degradation of multiple plastic types via previously identified PET degrading bacteria. Int. J. Mol. Sci. 23 (5612), http://dx.doi.org/10.3390/ijms23105612.

Faraca, G., Martinez-Sanchez, V., Astrup, T.F., 2019. Environmental life cycle cost assessment: Recycling of hard plastic waste collected at danish recycling centres. Resour. Conserv. Recycl. 143, 299–309. http://dx.doi.org/10.1016/j.resconrec.2019.01.014.

Ferrero, B., Attallah, O.A., Valera, M.Á., Aleksic, I., Azeem, M., Nikodinovic-Runic, J., Fournet, M.B., 2022. Rendering bio-inert low-density polyethylene amenable for biodegradation via fast high throughput reactive extrusion assisted oxidation. J. Polym. Environ. 30, 2837–2846. http://dx.doi.org/10.1007/s10924-022-02400-w.

Gambarini, V., Pantos, O., Kingsbury, J.M., Weaver, L., Handley, K.M., Lear, G., 2021. Phylogenetic distribution of plastic-degrading microorganisms. mSystems 6, e01112-20. http://dx.doi.org/10.1128/mSystems.01112-20.

Gangola, S., Joshi, S., Kumar, S., Sharma, B., Sharma, A., 2021. Differential proteomic analysis under pesticides stress and normal conditions in *Bacillus cereus* 2D. PLoS One 16, e0253106. http://dx.doi.org/10.1371/journal.pone.0253106.

Gangola, S., Sharma, A., Bhatt, P., Khati, P., Chaudhary, P., 2018. Presence of esterase and laccase in *Bacillus subtilis* facilitates biodegradation and detoxification of cypermethrin. Sci. Rep. 8, 12755. http://dx.doi.org/10.1038/s41598-018-31082-5.

Gangola, S., Sharma, A., Joshi, S., Bhandari, G., Prakash, O., Govarthanan, M., Kim, W., Bhatt, P., 2022. Novel mechanism and degradation kinetics of pesticides mixture using *Bacillus* sp. strain 3C in contaminated sites. Pest. Biochem. Physiol. 181, 104996. http://dx.doi.org/10.1016/j.pestbp.2021.104996.

Hanim, C., 2017. Effect of pH and temperature on *Bacillus subtilis* fncc 0059 oxalate decarboxylase activity. Pak. J. Biol. Sci. 20, 436–441. http://dx.doi.org/10.3923/pjbs.2017.436.441.

Holland, B.J., Hay, J.N., 2002. The thermal degradation of pet and analogous polyesters measured by thermal analysis–fourier transform infrared spectroscopy. Polymer 43, 1835–1847. http://dx.doi.org/10.1016/S0032-3861(01)00775-3.

Huang, X., Cao, L., Qin, Z., Li, S., Kong, W., Liu, Y., 2018. Tat-independent secretion of polyethylene terephthalate hydrolase petase in *Bacillus subtilis* 168 mediated by its native signal peptide. J. Agricult. Food Chem. 66, 13217–13227. http://dx.doi.org/10.1021/acs.jafc.8b05038.

Jaeger, K.-E., Kovacic, F., 2014. Determination of lipolytic enzyme activities. In: Pseudomonas Methods and Protocols. pp. 111–134. http://dx.doi.org/10.1007/978-1-4939-0473-0_12.

Jaiswal, S., Sharma, B., Shukla, P., 2020. Integrated approaches in microbial degradation of plastics. Environ. Technol. Innov. 17, 100567. http://dx.doi.org/10.1016/j.eti.2019.100567.

Jia, Y., Samak, N.A., Hao, X., Chen, Z., Wen, Q., Xing, J., 2022. Hydrophobic cell surface display system of petase as a sustainable biocatalyst for PET degradation. Front. Microbiol. 13, 1005480. http://dx.doi.org/10.3389/fmicb.2022.1005480.

Jones, P., Binns, D., Chang, H.-Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., 2014. Interproscan 5: Genome-scale protein function classification. Bioinformatics 30, 1236–1240. http://dx.doi.org/10.1093/bioinformatics/btu031.

Kawai, F., Kawabata, T., Oda, M., 2019. Current knowledge on enzymatic PET degradation and its possible application to waste stream management and other fields. Appl. Microbiol. Biotechnol. 103, 4253–4268. http://dx.doi.org/10.1007/s00253-019-09717-y.

Kenny, S.T., Runic, J.N., Kaminsky, W., Woods, T., Babu, R.P., Keely, C.M., Blau, W., O'Connor, K.E., 2008. Up-cycling of PET (polyethylene terephthalate) to the biodegradable plastic PHA (polyhydroxyalkanoate). Environ. Sci. Technol. 42, 7696–7701. http://dx.doi.org/10.1021/es801010e.

Kim, H.T., Kim, J.K., Cha, H.G., Kang, M.J., Lee, H.S., Khang, T.U., Yun, E.J., Lee, D.-H., Song, B.K., Park, S.J., 2019. Biological valorization of poly (ethylene terephthalate) monomers for upcycling waste PET. Sustain. Chem. Eng. 7, 19396–19406. http://dx.doi.org/10.1021/acssuschemeng.9b03908.

Kim, H.T., Ryu, M.Hee, Jung, Y.J., Lim, S., Song, H.M., Park, J., Hwang, S.Y., Lee, H.S., Yeon, Y.J., Sung, B.H., 2021. Chemo-biological upcycling of poly (ethylene terephthalate) to multifunctional coating materials. ChemSusChem 14, 4251–4259. http://dx.doi.org/10.1002/cssc.202100909.

Kosiorowska, K.E., Moreno, A.D., Iglesias, R., Leluk, K., Mirończuk, A.M., 2022. Production of petase by engineered Yarrowia lipolytica for efficient poly (ethylene terephthalate) biodegradation. Sci. Total Environ. 846, 157358. http://dx.doi.org/10.1016/j.scitotenv.2022.157358.

Kumari, A., Bano, N., Bag, S.K., Chaudhary, D.R., Jha, B., 2021. Transcriptome-guided insights into plastic degradation by the marine bacterium. Front. Microbiol. 2761.

Kurutos, A., Ilic-Tomic, T., Kamounah, F.S., Vasilev, A.A., Nikodinovic-Runic, J., 2020. Non-cytotoxic photostable monomethine cyanine platforms: Combined paradigm of nucleic acid staining and in vivo imaging. J. Photochem. Photobiol. A 397, 112598. http://dx.doi.org/10.1016/j.jphotochem.2020.112598.

Laskar, N., Kumar, U., 2019. Plastics and microplastics: A threat to environment. Environ. Technol. Innov. 14, 100352. http://dx.doi.org/10.1016/j.eti.2019.100352.

Li, W., O'Neill, K.R., Haft, D.H., DiCuccio, M., Chetvernin, V., Badretdin, A., Coulouris, G., Chitsaz, F., Derbyshire, M.K., Durkin, A.S., 2021. Refseq: Expanding the prokaryotic genome annotation pipeline reach with protein family model curation. Nucleic Acids Res. 49, D1020–D1028. http://dx.doi.org/10.1093/nar/gkaa1105.

Liya, S.M., Umesh, M., Nag, A., Chinnathambi, A., Alharbi, S.A., Jhanani, G.K., Shanmugam, S., Brindhadevi, K., 2023. Optimized production of keratinolytic proteases from Bacillus tropicus ls27 and its application as a sustainable alternative for dehairing, destaining and metal recovery. Environ. Res. 221, 115283. http://dx.doi.org/10.1016/j.envres.2023.115283.

Magalhães, R.P., Cunha, J.M., Sousa, S.F., 2021. Perspectives on the role of enzymatic biocatalysis for the degradation of plastic PET. Int. J. Mol. Sci. 22 (11257), http://dx.doi.org/10.3390/ijms222011257.

Maheswaran, B., Al-Ansari, M., Al-Humaid, L, Raj, J.S., Kim, W., Karmegam, N., Rafi, K.M., 2023. In vivo degradation of polyethylene terephthalate using microbial isolates from plastic polluted environment. Chemosphere 310, 136757. http://dx.doi.org/10.1016/j.chemosphere.2022.136757.

Makkam, S., Harnnarongchai, W., 2014. Rheological and mechanical properties of recycled PET modified by reactive extrusion. Energy Procedia 56, 547–553. http://dx.doi.org/10.1016/j.egypro.2014.07.191.

Manni, M., Berkeley, M.R., Seppey, M., Simão, F.A., Zdobnov, E.M., 2021. Busco update: Novel and streamlined workflows along with broader and deeper phylogenetic coverage for scoring of eukaryotic, prokaryotic, and viral genomes. Mol. Biol. Evol. 38, 4647–4654. http://dx.doi.org/10.1093/molbev/msab199.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet. J. 17, 10–12. http://dx.doi.org/10.14806/ej.17.1.200.

Meyer-Cifuentes, I.E., Öztürk, B., 2021. Mle046 is a marine mesophilic mhetase-like enzyme. Front. Microbiol. 12, 693985. http://dx.doi.org/10.3389/fmicb.2021.693985.

Mohanan, N., Montazer, Z., Sharma, P.K., Levin, D.B., 2020. Microbial and enzymatic degradation of synthetic plastics. Front. Microbiol. 11, 580709. http://dx.doi.org/10.3389/fmicb.2020.580709.

Molitor, R., Bollinger, A., Kubicki, S., Loeschcke, A., Jaeger, K.E., Thies, S., 2020. Agar plate-based screening methods for the identification of polyester hydrolysis by Pseudomonas species. Microbiol. Biotechnol. 13, 274–284. http://dx.doi.org/10.1111/1751-7915.13418.

Mrigwani, A., Thakur, B., Guptasarma, P., 2022. Conversion of polyethylene terephthalate into pure terephthalic acid through synergy between a solid-degrading cutinase and a reaction intermediate-hydrolysing carboxylesterase. Green Chem. 24, 6707–6719. http://dx.doi.org/10.1039/D2GC01965E.

Nguyen, L.H., Nguyen, B.-S., Le, D.-T., Alomar, T.S., AlMasoud, N., Ghotekar, S., Oza, R., Raizada, P., Singh, P., Nguyen, V.-H., 2023. A concept for the biotechnological minimizing of emerging plastics, micro- and nano-plastics pollutants from the environment: A review. Environ. Res. 216, 114342. http://dx.doi.org/10.1016/j.envres.2022.114342.

Nikolaivits, E., Taxeidis, G., Gkountela, C., Vouyiouka, S., Maslak, V., Nikodinovic-Runic, J., Topakas, E., 2022. A polyesterase from the antarctic bacterium Moraxella sp. Degrades highly crystalline synthetic polymers. J. Hazard. Mater. 434, 128900. http://dx.doi.org/10.1016/j.jhazmat.2022.128900.

Perera-Costa, D., Bruque, J.M., González-Martín, M.L., Gómez-García, A.C., Vadillo-Rodríguez, V., 2014. Studying the influence of surface topography on bacterial adhesion using spatially organized microtopographic surface patterns. Langmuir 30, 4633–4641. http://dx.doi.org/10.1021/la5001057.

PlasticsEurope, 2021. Plastics - the facts 2021. https://plasticseurope.org/knowledge-hub/plastics-the-facts-2021/. (Accessed 1 February 2023).

Qi, X., Ma, Y., Chang, H., Li, B., Ding, M., Yuan, Y., 2021. Evaluation of PET degradation using artificial microbial consortia. Front. Microbiol. 12, 778828. http://dx.doi.org/10.3389/fmicb.2021.778828.

Qiu, L, Yin, X., Liu, T., Zhang, H., Chen, G., Wu, S., 2020. Biodegradation of bis (2-hydroxyethyl) terephthalate by a newly isolated Enterobacter sp. Hy1 and characterization of its esterase properties. J. Basic Microbiol. 60, 699–711. http://dx.doi.org/10.1002/jobm.202000053.

Ragaert, K., Delva, L., Van Geem, K., 2017. Mechanical and chemical recycling of solid plastic waste. Waste Manag. 69, 24–58. http://dx.doi.org/10.1016/j.wasman.2017.07.044.

Ribitsch, D., Heumann, S., Trotscha, E., Herrero Acero, E., Greimel, K., Leber, R., Birner-Gruenberger, R., Deller, S., Eiteljoerg, I., Remler, P., 2011. Hydrolysis of polyethyleneterephthalate by p-nitrobenzylesterase from Bacillus subtilis. Biotechnol. Prog. 27, 951–960. http://dx.doi.org/10.1002/btpr.610.

Roberts, C., Edwards, S., Vague, M., León-Zayas, R., Scheffer, H., Chan, G., Swartz, N.A., Mellies, J.L., 2020. Environmental consortium containing Pseudomonas and Bacillus species synergistically degrades polyethylene terephthalate plastic. Msphere 5, e01151-20. http://dx.doi.org/10.1128/mSphere.01151-20.

Samak, N.A., Jia, Y., Sharshar, M.M., Mu, T., Yang, M., Peh, S., Xing, J., 2020. Recent advances in biocatalysts engineering for polyethylene terephthalate plastic waste green recycling. Environ. Int. 145, 106144. http://dx.doi.org/10.1016/j.envint.2020.106144.

Sammon, C., Yarwood, J., Everall, N., 2000. A FTIR-AIR study of liquid diffusion processes in PET films: Comparison of water with simple alcohols. Polymer 41, 2521–2534. http://dx.doi.org/10.1016/S0032-3861(99)00405-X.

Shah, Z., Gulzar, M., Hasan, F., Shah, A.A., 2016. Degradation of polyester polyurethane by an indigenously developed consortium of Pseudomonas and Bacillus species isolated from soil. Polym. Degrad. Stab. 134, 349–356. http://dx.doi.org/10.1016/j.polymdegradstab.2016.11.003.

Shah, Z., Krumholz, L., Aktas, D.F., Hasan, F., Khattak, M., Shah, A.A., 2013. Degradation of polyester polyurethane by a newly isolated soil bacterium, Bacillus subtilis strain MZA-75. Biodegradation 24, 865–877. http://dx.doi.org/10.1007/s10532-013-9634-5.

Sharma, R., Jasrotia, T., Umar, A., Sharma, M., Sharma, S., Kumar, R., Alkhanjaf, A.A.M., Vats, R., Beniwal, V., Kumar, R., Singh, J., 2022. Effective removal of Pb(ii) and Ni(ii) ions by Bacillus cereus and Bacillus pumilus: An experimental and mechanistic approach. Environ. Res. 212, 113337. http://dx.doi.org/10.1016/j.envres.2022.113337.

Skariyachan, S., Setlur, A.S., Naik, S.Y., Naik, A.A., Usharani, M., Vasist, K.S., 2017. Enhanced biodegradation of low and high-density polyethylene by novel bacterial consortia formulated from plastic-contaminated cow dung under thermophilic conditions. Environ. Sci. Pollut. Res. 24, 8443–8457. http://dx.doi.org/10.1007/s11356-017-8537-0.

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Souza, C.C.d., Guimarães, J.M., Pereira, S.d.S., Mariúba, L.A.M., 2021. The multifunctionality of expression systems in *Bacillus subtilis*: Emerging devices for the production of recombinant proteins. Exp. Biol. Med. 246, 2443–2453. http://dx.doi.org/10.1177/15353702211030189.

Sulaiman, S., Yamato, S., Kanaya, E., Kim, J.-J., Koga, Y., Takano, K., Kanaya, S., 2012. Isolation of a novel cutinase homolog with polyethylene terephthalate-degrading activity from leaf-branch compost by using a metagenomic approach. Appl. Environ. Microbiol. 78, 1556–1562. http://dx.doi.org/10.1128/AEM.06725-11.

Suzuki, G., Uchida, N., Tanaka, K., Matsukami, H., Kunisue, T., Takahashi, S., Viet, P.H., Kuramochi, H., Osako, M., 2022. Mechanical recycling of plastic waste as a point source of microplastic pollution. Environ. Pollut. 303, 119114. http://dx.doi.org/10.1016/j.envpol.2022.119114.

Teufel, F., Almagro Armenteros, J.J., Johansen, A.R., Gíslason, M.H., Pihl, S.I., Tsirigos, K.D., Winther, O., Brunak, S., von Heijne, G., Nielsen, H., 2022. Signalp 6.0 predicts all five types of signal peptides using protein language models. Nature Biotechnol. 40 (7), 1023–1025. http://dx.doi.org/10.1038/s41587-021-01156-3.

Thomsen, T.B., Hunt, C.J., Meyer, A.S., 2022. Influence of substrate crystallinity and glass transition temperature on enzymatic degradation of polyethylene terephthalate (PET). New Biotechnol. 69, 28–35. http://dx.doi.org/10.1016/j.nbt.2022.02.006.

Tiso, T., Narancic, T., Wei, R., Pollet, E., Beagan, N., Schröder, K., Honak, A., Jiang, M., Kenny, S.T., Wierckx, N., 2021. Towards bio-upcycling of polyethylene terephthalate. Metab. Eng. 66, 167–178. http://dx.doi.org/10.1016/j.ymben.2021.03.011.

Tournier, V., Topham, C., Gilles, A., David, B., Folgoas, C., Moya-Leclair, E., Kamionka, E., Desrousseaux, M.-L., Texier, H., Gavalda, S., 2020. An engineered PET depolymerase to break down and recycle plastic bottles. Nature 580, 216–219. http://dx.doi.org/10.1038/s41586-020-2149-4.

Uekert, T., DesVeaux, J.S., Singh, A., Nicholson, S.R., Lamers, P., Ghosh, T., McGeehan, J.E., Carpenter, A.C., Beckham, G.T., 2022. Life cycle assessment of enzymatic poly (ethylene terephthalate) recycling. Green Chem. 24, 6531–6543. http://dx.doi.org/10.1039/D2GC02162E.

Wang, N., Guan, F., Lv, X., Han, D., Zhang, Y., Wu, N., Xia, X., Tian, J., 2020. Enhancing secretion of polyethylene terephthalate hydrolase petase in *Bacillus subtilis* WB600 mediated by the SP_{amy} signal peptide. Lett. Appl. Microbiol. 71, 235–241. http://dx.doi.org/10.1111/lam.13312.

Wang, D., Lin, J., Lin, J., Wang, W., Li, S., 2019. Biodegradation of petroleum hydrocarbons by *Bacillus subtilis* BL-27, a strain with weak hydrophobicity. Molecules 24 (3021), http://dx.doi.org/10.3390/molecules24173021.

Wei, R., Breite, D., Song, C., Gräsing, D., Ploss, T., Hille, P., Schwerdtfeger, R., Matysik, J., Schulze, A., Zimmermann, W., 2019. Biocatalytic degradation efficiency of postconsumer polyethylene terephthalate packaging determined by their polymer microstructures. Adv. Sci. 6, 1900491. http://dx.doi.org/10.1002/advs.201900491.

Wei, R., Zimmermann, W., 2017. Biocatalysis as a green route for recycling the recalcitrant plastic polyethylene terephthalate. Microb. Biotechnol. 10, 1302. http://dx.doi.org/10.1111/1751-7915.12714.

Wunderlich, B., 1973. Macromolecular Physics. vol. I: Crystal Structure, Morphology, Defects Summary. pp. 178–379. http://dx.doi.org/10.1016/B978-0-12-765601-4.X5001-X.

Xi, X., Ni, K., Hao, H., Shang, Y., Zhao, B., Qian, Z., 2021. Secretory expression in *Bacillus subtilis* and biochemical characterization of a highly thermostable polyethylene terephthalate hydrolase from bacterium hr29. Enzyme Microbial Technol. 143, 109715. http://dx.doi.org/10.1016/j.enzmictec.2020.109715.

Yang, Y., Malten, M., Grote, A., Jahn, D., Deckwer, W.D., 2007. Codon optimized *Thermobifida fusca* hydrolase secreted by *Bacillus megaterium*. Biotechnol. Bioeng. 96, 780–794. http://dx.doi.org/10.1002/bit.21167.

Yi, Y., Kuipers, O.P., 2017. Development of an efficient electroporation method for *Rhizobacterial bacillus* mycoides strains. J. Microbiol. Meth. 133, 82–86. http://dx.doi.org/10.1016/j.mimet.2016.12.022.

Yoshida, S., Hiraga, K., Takehana, T., Taniguchi, I., Yamaji, H., Maeda, Y., Toyohara, K., Miyamoto, K., Kimura, Y., Oda, K., 2016. A bacterium that degrades and assimilates poly (ethylene terephthalate). Science 351, 1196–1199. http://dx.doi.org/10.1126/science.aad6359.

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1.2. Set of small molecule polyurethane (PU) model substrates: Ecotoxicity evaluation and identification of PU degrading biocatalysts

Article

Set of Small Molecule Polyurethane (PU) Model Substrates: Ecotoxicity Evaluation and Identification of PU Degrading Biocatalysts

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Abstract: Polyurethanes (PUs) are an exceedingly heterogeneous group of plastic polymers, widely used in a variety of industries from construction to medical implants. In the past decades, we have witnessed the accumulation of PU waste and its detrimental environmental impacts. PUs have been identified as one of the most toxic polymers leaching hazardous compounds derived both from the polymer itself and the additives used in production. Further environmental impact assessment, identification and characterization of substances derived from PU materials and establishing efficient degradation strategies are crucial. Thus, a selection of eight synthetic model compounds which represent partial PU hydrolysis products were synthesized and characterized both in terms of toxicity and suitability to be used as substrates for the identification of novel biocatalysts for PU biodegradation. Overall, the compounds exhibited low in vitro cytotoxicity against a healthy human fibroblast cell line and virtually no toxic effect on the nematode *Caenorhabditis elegans* up to 500 µg mL^{-1}, and two of the substrates showed moderate aquatic ecotoxicity with EC_{50} values 53 µg mL^{-1} and 45 µg mL^{-1}, respectively, on *Aliivibrio fischeri*. The compounds were successfully applied to study the mechanism of ester and urethane bond cleaving preference of known plastic-degrading enzymes and were used to single out a novel PU-degrading biocatalyst, *Amycolatopsis mediterranei* ISP5501, among 220 microbial strains. *A. mediterranei* ISP5501 can also degrade commercially available polyether and polyester PU materials, reducing the average molecular number of the polymer up to 13.5%. This study uncovered a biocatalyst capable of degrading different types of PUs and identified potential enzymes responsible as a key step in developing biotechnological process for PU waste treatment options.

Keywords: polyurethane; biocatalysis; model substrate; ecotoxicology; *Amycolatopsis mediterranei*; biodegradation; bioremediation

1. Introduction

Polyurethanes (PUs) are ranked the sixth most common synthetic polymer used [1], with a growing market value estimate of over USD 50 billion for 2021 [2]. The production of PUs involves reacting diisocyanates with polyols to obtain thermoplastics, thermosets, or foams [3]. PUs are widely used as coatings, insulators, foams, elastic fibers, textiles

in carpet underlayment, thermal isolation, car seats, mattresses, etc. [4]. High and wide usage leads to waste accumulation, which more than often ends up in landfills and the environment. The bulk of PU waste is incinerated for energy recovery and releasing toxic compounds [5], while 29.7% is recycled and 30.8% is still landfilled [6]. PU and PU microplastic particles are identified as one of the more toxic polymers [7,8]. Widely used PU mattresses have been shown to continually release a number of volatile organic compounds [9]. Thermal recycling of mattresses also releases toxic isocyanates [10] while PU coatings leach ecotoxic compounds based on 4,4'-methylenediphenyl diisocyanate (4,4'-MDI) and toluene diisocyanate (TDI), which are known carcinogens [11]. On the other hand, certain types of PU are well established for use as medical devices, with numerous studies confirming their biocompatibility and non-toxicity [12], thus underlining the great diversity of PU polymers.

Current recycling strategies for PU waste revolve around mechanical and chemical recycling. Mechanical approaches transform PU waste into granules, flakes, or powder, which can be used be used in new products, i.e., PU carpet underlayment [13]. It is a fairly cost-effective and environmentally acceptable process; however, it is a form of 'downcycling' as materials of lower quality and value are produced. For the recovery of monomers that can be incorporated into new polymers, chemical recycling is a more sensible route. Hydrolysis, alcoholysis, aminolysis, phosphorolysis [6], and glycolysis [14] have been used in the recycling of PU in pilot-scale plants [13]. However, the high energy consumption and environmental impacts of chemical recycling decrease its role in sustainable development; however, some of these problems could be bypassed by biocatalysis. The benefits of biocatalysis have clearly been shown in the case of polyethylene terephthalate (PET), which has led to the development of biotechnological depolymerization of post-consumer PET bottles with the efficient recovery of terephthalic acid [15]. Therefore, research efforts to develop superior enzymes and modify microorganisms for the degradation of other plastic and mixed plastic waste materials are ongoing. Based on the type of polyols used, PUs are classified into polyester PUs and polyether PUs. Depending on the type of diisocyanate used, PUs can be aromatic or aliphatic [16]. The structure of thermoplastic PU polymers consists of hard (highly crystalline regions made up of isocyanates, chain extenders with urethane bonds) and soft segments (amorphous regions mainly consisting of polyols with ester or ether bonds) [17] (Figure 1a).

Hard/soft segment composition and isocyanate type govern the biodegradability of PU, with polyester Pus being more prone to microbial degradation than polyether Pus. Soft segments are more accessible for enzymatic attack, ester bonds have higher biodegradability than urethane bonds [18], and aromatic isocyanate-based PUs are considered more difficult to biodegrade than the aliphatic ones [17]. Despite decades of research, an efficient biocatalytic system for PU hydrolysis has not been reported yet [1,19]. From a number of microorganisms reported to degrade PU, only a handful of enzymes/microorganisms have been shown to hydrolyze the urethane bond [20]. A database of known and confirmed plastic-degrading enzymes—Plastic-Active Enzymes Database (PAZy) [21,22]—contains 10 PU-active enzymes; however, most of them act only on the ester bonds of the polymer [23]. Given the complex structure and variety of bonds that can be found in PU, the research focus is shifting from individual enzymes to multiple enzyme systems. One such study employed an amidase and esterase in a bid to simultaneously degrade urethane and ester linkages in four different thermoplastic PUs and proved that combining enzymes leads to increased urethane bond hydrolysis [24]. Alternative approaches, such as using *Tenebrio molitor* larvae and their gut microbiota for the degradation of PU, have been employed as well [25]. However, the lack of efficient and robust urethane bond degrading biocatalysts is still the main bottleneck in the development of biotechnological PU waste recycling systems [26].
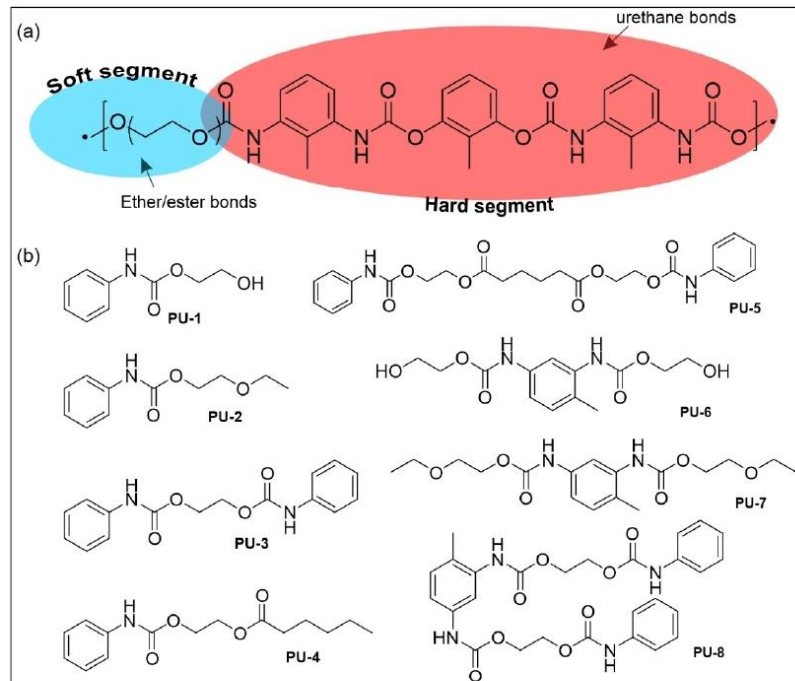
Figure 1. Polyurethane (PU) (**a**) polymer representation; (**b**) proposed model substrates synthesized in this study derived from hydrolysis of PU hard segment and PU-related compounds for detection of novel biocatalysts.

The most widespread substrate for assessing PU degradation is a colloidal dispersion, Impranil, an anionic aliphatic polyester PU of proprietary structure [27]. A change in optical density (clearing), whether in solution, on agar plates, or combined with dyes, is considered evidence of PU degradation [28]. Impranil-clearing assays cannot give information on the type of enzyme responsible for the activity and whether ether or urethane bonds were hydrolyzed, and, most importantly, the amount of Impranil clearing does not always correlate to the amount of degradation [29]. However, Impranil has proved useful in research describing the adsorption of peptides to plastic [30]. When investigating enzyme mechanisms, urethane bond-containing small molecules present far more promising approaches with *p*-toluenesulfonamide- [24] and *p*-nitrophenol- [31] tagged molecules being used for assessing urethanase activity.

In this work, we synthesized eight PU model substrates based on widely used phenyl isocyanate and toluene diisocyanate containing both urethane and ester bonds (Figure 1b). These substrates were used for the screening and identification of novel PU-degrading biocatalysts while allowing for the study of the mechanism of the action. PU model substrates were designed to represent polymer partial degradation products, valuable for assessing potential environmental risks associated with PU materials and their degradation. Accordingly, their toxicity and environmental impact were investigated and compared to known PU-associated pollutants, including adipic acid and 2,4- toluenediamine (2,4-TDA). *Amycolatopsis mediterranei* ISP5501 was identified as a urethane bond-degrading strain using the PU-7 model substrate and was further confirmed to degrade both polyester and polyether PU materials. Potential enzymes involved were identified by the genome analysis.

2. Results and Discussion

2.1. PU-Model Compounds Synthesis and Characterization

Based on the structural characteristics of different PU materials, we designed eight PU model compounds (Figure 1b). Six of them were synthesized and structurally characterized for the first time (PU-2, PU-3, PU-4, PU-5, PU-6, and PU-8), while two of them were reported earlier (PU-1 and PU-7), but lacked proper spectral characterization [32–34]. NMR spectra of the full set of PU model compounds are provided as Figures S1–S16.

Model substrates were synthesized following three main reactions: (1) reaction of hydroxyalkyl esters with phenyl isocyanate and toluene diisocyanate; (2) reaction of 2-hydroxyethyl phenyl-carbamate with hexanoyl chloride or adipic chloride; and (3) hydrogenolysis. Compounds PU-1, PU-2, and PU-3 were obtained by reaction of hydroxyalkyl esters with phenyl isocyanate, while compounds PU-6, PU-7, and PU-8 were obtained by reaction of hydroxyalkyl esters with toluene diisocyanate. These reactions are typical reactions of obtaining so-called urethane ester monomers whose polymerization gives polymeric materials applicable as impregnating and adhesion agents, crosslinking agents, non-toxic dental materials, coatings, and waveguide protection materials [35]. Compounds PU-4 and PU-5 were obtained by reacting 2-hydroxyethyl phenyl-carbamate and hexanoyl chloride or adipic chloride. The yield of all products was highly dependent on the reaction conditions and required optimization of the purification process to achieve both high yield and purity. Solubility in a selection of common organic solvents was tested for each of the PU model compounds, with all being well soluble in DMSO as solvent of choice for toxicity evaluations (Table S1). As expected, PU-8 was the least soluble, especially in polar solvents, including methanol and ethanol. PU-6, PU-7, and PU-8 represent urethane bond-containing partial degradation products of TDI-based PUs with different polyol segments (Figure 1b) and are realistic targets for further enzyme degradation and substrates for urethanase enzyme detection. PU-4 and PU-5 containing both urethane and ester bonds were used for the study of enzyme cleaving preferences.

The biodegradability of urethane bonds in small molecules is higher than in PU polymers [1], but the diverse structures of PU model substrates allow for examining urethane bond cleaving in different molecular contexts and thus bridge the gap from small molecule model substrates to the bulk polymer hydrolysis. Previously, a model substrate composed of 14 units of adipic acid, seven units of 1,4-butanediol (BDO), seven units of ethylene glycol (EG), and one unit of 2,4-TDA was used for assessment of microbial PU hydrolysis and subsequent monomer utilization [36].

2.2. Cyto- and Ecotoxicity of the PU-Model Compounds

The cytotoxicity of carbamate compounds is well documented in the literature [37]; therefore, the antiproliferative effect of PU model substrates was tested on a healthy lung fibroblast MRC-5 cell line as the inhalation may be the route of entry for compounds released during polyurethane degradation [38]. Low toxicity of the compounds was observed under the conditions tested. Even at a relatively high concentration of 10 µg mL^{-1}, all tested compounds supported 90% to 100% cell survival, while in higher concentrations for compounds PU-3, PU-6, PU-7, and PU-8, cell survival rates were between 60% to 80% (Figure 2a). For compounds PU-3, PU-6, and PU-8, greater cell death may be caused by physical interference with cells, as they showed lower solubility and were in a state of suspension when added to aqueous solution of cell propagation medium.
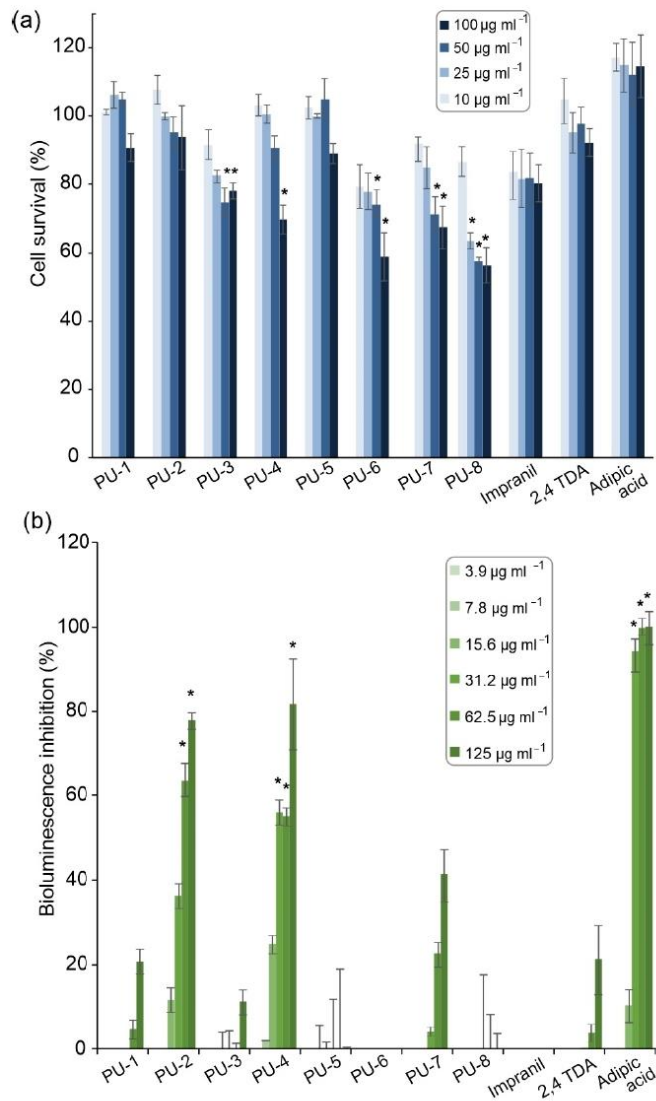
Figure 2. Quantification of in vitro MRC-5 cells survival in the presence of PU model compounds at concentration range (**a**). Inhibitory effect of PU model substrates on *A. fischeri* bioluminescence at concentration range (**b**). The effect was compared to untreated control using a *t*-test, * $p \leq 0.01$.

As a model system for ecotoxicity evaluation of organic chemicals, *A. fischeri* is usually employed for the evaluation of aquatic toxicity to marine and freshwater organisms. The inhibition of bioluminescence is a non-specific and sensitive toxicity assay [39]. PU-5, PU-6, and PU-8 displayed no inhibitory effects on *A. fischeri* bioluminescence, while PU-1 and PU-3 caused only a minimal decrease in bioluminescence at the highest concentration tested and can be considered non-toxic as well. Comparing all investigated PU model compounds, PU-2 and PU-4 proved to have the highest aquatic toxicity, with EC_{50} values

of 53 µg mL^{-1} and 45 µg mL^{-1}, respectively (Figure 2b), and with EC$_{50}$ values between 100 and 10 µg mL^{-1} could be considered as moderately toxic [40]. In contrast, the EC$_{20}$ value of 2,4-TDA was 116 µg mL^{-1}, with even lower concentrations reported in the literature (50 µg mL^{-1}) [41]. These results indicate that PU degradation intermediates can be more toxic than diamines released during complete PU hydrolysis and need to be taken into account when assessing the environmental impact of PU degradation strategies. Adipic acid, another product of partial PU hydrolysis, had the lowest EC$_{50}$ value (18 µg mL^{-1}) and was the only one to completely inhibit *A. fischeri* bioluminescence at higher concentrations. To the best of our knowledge, there are no reports investigating the influence of adipic acid on *A. fischeri,* with Šepič et al. reporting EC$_{50}$ values of 140 and 128 µg mL^{-1} after acute tests with crustacea *Daphnia magna* and *Thamnocephalus platyurus*, respectively [42]. PU and polyvinyl chloride (PVC) leachates of unknown composition were previously found toxic to *D. magna* [43].

C. elegans is a multicellular model organism that combines both the advantages of using whole animals and in vitro systems for toxicity assessment, with comparative studies confirming the replicability of results in mammalian model organisms [44]. Given its terrestrial habitat and ubiquitous distribution, *C. elegans* may also prove useful in investigating the environmental impact of plastic degradation products [45]. The PU model substrates did not cause *C. elegans* death in any of the concentrations tested (results not shown). Only TDA at a concentration of 500 µg mL^{-1} caused 70% mortality; however, it is highly unlikely that TDA can reach such high concentrations in the environment.

Worth mentioning is the fact that Impranil as polymeric dispersion proved to be less toxic than monomeric PU degradation products in all toxicity tests, underlining the need for toxicity analysis when investigating and optimizing polymer degradation processes. In a study exploring the baseline toxicity, oxidative stress induction, cytotoxicity, and endocrine activity of plastic extracts, PUs were identified as having the highest toxicity along with poly(vinyl chloride) (PVC) [7]. Other studies explored the toxicity of specific PU degradation products, such as the corresponding diamines derived from two of the most common diisocyanates, with both MDA [46] and TDA [36,47] classified as carcinogens.

2.3. Degradation of PU Model Substrates by Known Esterases and Proteases

The label-free nature of the PU model substrates allows for the examination of the enzyme mechanisms without potential enzyme bias towards a chromophore of fluorophore [48]. The applicability of such a set of substrates was demonstrated for the screening and characterization of novel PETases [49] and the identification of bisphenol-A polycarbonate-degrading bacteria [50]. The potential of PU model substrates to be used as bond-specific screening molecules was assessed using hydrolases from different families. Recombinant FoCut5a, HiC, and IsPETase are already proven to be capable of cleaving ester bonds of different polyester materials. Specifically, FoCut5a can degrade PET model substrates and polycaprolactone (PCL) powder (Dimarogona et al., 2015), and HiC cutinase can fully degrade PET films of low crystallinity (Ronkvist et al., 2009), while IsPETase is a well-known PET hydrolase (Yoshida et al., 2016). Recombinant DaPUase was from the bacterium *Comamonas acidovorans*, a microorganism able to utilize polyester PU as the sole carbon source (Akutsu et al., 1998). Lastly, the two commercial proteases (BacProt and StrepProt) were chosen because they can potentially act on both ester and urethane bonds. DaPUase, FoCut5a, and IsPETase are enzymes that show maximum activity at temperatures around 30 °C, in contrast to BacProt, HiC, and StrepProt with temperature optimum around 50 °C. As a result, the hydrolysis of PU-5 compound was analyzed after treatment with both esterases and proteases at 30 °C and at 50 °C (Figure S17). PU-5, a model substrate containing two terminal phenyl groups (Figure 1b), was chosen as the model substrate since the degradation could be efficiently monitored via a standard HPLC equipped with a UV-detector.

The main hydrolysis product after treating PU-5 with FoCut5a, IsPETase, HiC, and DaPUase was PU-1, indicative of ester bond cleaving (Figure S17a). Smaller amounts of

PU-4 were also detected, further confirming that the ester bond was cleaved since both PU-1 and PU-4 are products of ester bond cleavage. The tested enzymes could not recognize the urethane bond under conditions tested, probably because of the stereochemical inhibition caused by the aromatic ring. In the sample containing no enzyme, an additional product was detected, which can be attributed to autohydrolysis of the substrate during incubation. Interestingly, protease digestion also resulted in PU-1 as the main degradation product, cleaving only the ester and not the urethane bond of the substrate. Serine proteases have been already reported to hydrolyze PU, showing simultaneously proteolytic and esterase activity [51]. BacProt is classified as subtilase, which belongs to the serine proteinase family, while StrepProt is a mixture of at least three proteases, including an extracellular serine protease. It seems that even these proteases show preference towards the ester bond over the urethane bond (Figure S17b). It has been reported that subtilisin and subtilisin-like proteases preferentially cleave hydrophobic non-aromatic residues [52].

2.4. Identification of New PU-Degrading Biocatalysts

A total of 220 microbial strains were screened for their Impranil-clearing activity on agar plates. Eighteen strains showed good zones of Impranil clearance (Figure 3) and were identified by 16S rDNA sequencing (Table S3), with the majority of strains showing the highest sequence similarity to *Streptomyces* species, a genus whose biocatalytic potential has gained interest in recent years [53]. The unknown structure of Impranil complicates any effort to investigate enzyme mechanisms using this substrate, but it does have a very important role in high-throughput pre-screening efforts [54]. Three strains with the ability to degrade Impranil, namely *Streptomyces* sp. TIT2, *Pseudomonas* sp. 44, and *Amycolatopsis mediterranei* ISP5501—and one *Bacillus* sp. BPM12, which had no Impranil-clearing activity but showed high esterase activity in standard assays (unpublished data)—were selected for biocatalytic reactions using PU-7 as a substrate (substrate with two urethane bonds at positions two and four; Figure 1b).
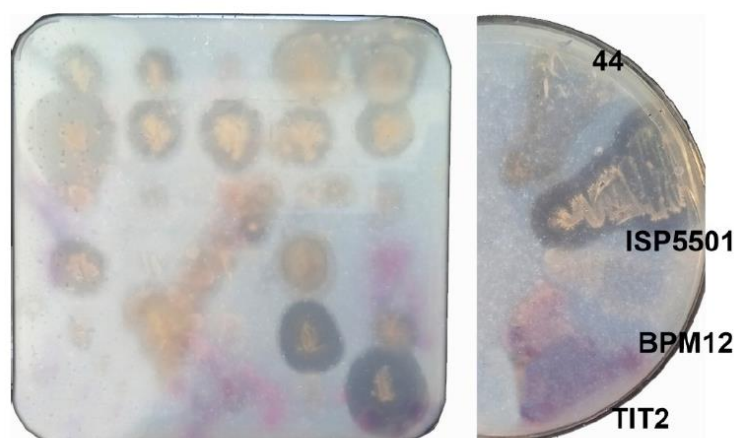
Figure 3. Pre-screening of bacterial strains on Impranil-containing agar plates. Based on the clearing zone, four bacterial strains (*Pseudomonas* sp. (44), *Amycolatopsis mediterranei* (ISP5501), *Bacillus* sp. (BPM12), and *Streptomyces* sp. (TIT2)) were selected for whole-cell biocatalytic reactions with PU-7.

Possible degradation products were not easily observed using a standard HPLC approach, therefore UHPLC-MS screen of whole-cell biotransformation reactions afforded the detection of urethanase activity in a high-throughput manner and to differentiate cleaving activities of amidases/proteases and esterases. MS spectra of reactions were compared to PU model substrate control reactions containing no biocatalyst (Figure 4).

Cells of *A. mediterraneiei* ISP5501 were the only ones to produce a variety of expected degradation products (Table S2, Figure S18). Masses of ten predicted urethane bond hydrolysis products were detected when *A. mediterraneiei* ISP5501 was used as a biocatalyst (Figure 4, Table S2). Both the aromatic and corresponding aliphatic hydrolysis products were detected. TDA and smaller aliphatic degradation products were not detected, either because they were near the detection limit (<100 *m/z*) or because they could have been used as carbon and energy source and assimilated by ISP5501 during biocatalysis. Based on the detected masses, the urethane bond could have been cleaved by two proposed methods. A distinction between cleaving the urethane bond in the amide vs. the ester part could be made since both amine and carbamic acid moiety-containing degradation products were detected. Based on this, ISP5501 could harbor more than one urethane-cleaving enzyme since urethane bond cleaving by esterase leads to the formation of carbamic acid (Figure 4a), while amidases/proteases hydrolysis yields amides (Figure 4b,c). The products of the activity of both types of enzymatic activities were also detected (Figure 4d,e).
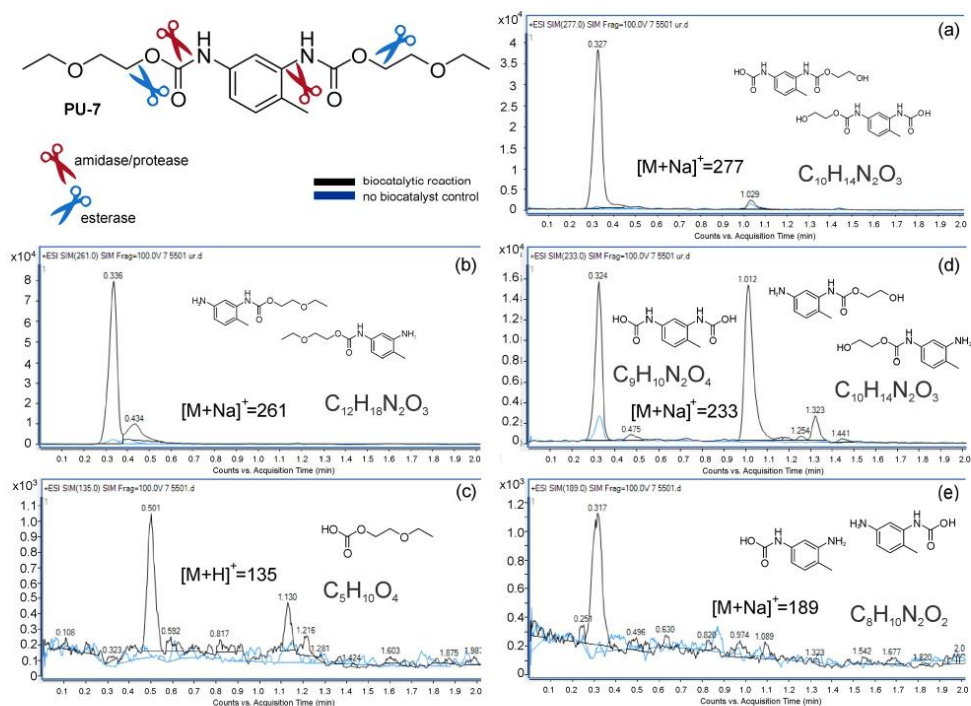
Figure 4. Biocatalytic reactions using PU-7 as substrate and resting whole cells of *A. mediterranei* ISP5501 as biocatalyst. Control reactions containing no biocatalysts are presented as a blue line. Products were identified using MS. The esterase activity generated two compounds of the molecular formula $C_{10}H_{14}N_2O_3$ (**a**), the amidase activity generated two compounds of the molecular formula $C_{12}H_{18}N_2O_3$ (**b**), and one compound of the molecular formula $C_5H_{10}O_4$ (**c**), while the activity of both types of enzymes generated two compounds of molecular formula $C_{10}H_{14}N_2O_3$ and one of $C_9H_{10}N_2O_4$ (**d**) and two compounds of the molecular formula $C_8H_{10}N_2O_2$ (**e**).

The proposed mechanisms of urethane bond cleavage by esterases in literature are conflicting. Liu et al. suggest the formation of a carbamic acid and an alcohol [1], while Magnin et al. argue that urethane bond cleaving will result in an amide and an alcohol with the release of a carbon dioxide molecule due to the instability of carbamic acid [23].

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Our results suggest urethane bond cleaving with esterases does produce stable carbamates that can be detected by MS. Furthermore, it is interesting to note that masses of ether bond hydrolysis products were detected as well, opening up the opportunity for using ISP5501 in the degradation of highly recalcitrant polyether PU.

A. mediterranei, traditionally linked with medicinal importance and industrial-scale production of rifamycin [55], is a taxon with considerable presence in polymer degradation studies. Research regarding the applicability of *Amycolatopsis* species in plastic degradation identified several efficient poly(L-lactic acid) (PLA) depolymerases [56] and a study exploring the phylogenetic distribution of plastic-degrading enzymes identified that the largest number of PLA depolymerases come from *Amycolatopsis* genus [57]. More than 10 PETase-like enzymes have also been identified in *Amycolatopsis* species [58], making this genus a promising source of novel plastic-degrading biocatalysts and a tool for plastic waste management in a circular economy.

2.5. PU Polymer Degradation by A. mediterranei ISP5501

The potential of the *A. mediterranei* ISP5501 to degrade PU polymer was further studied on different PU materials. Using whole cells as biocatalysts, the average molecular number Mn of polyether PU decreased by $7.0 \pm 0.9\%$, while in the presence of Impranil the Mn was reduced by $13.5 \pm 0.3\%$ compared to those of the initial untreated material (Table 1). The increased degradation observed in the second case can be attributed to the fact that hydrolytic enzymes such as esterases, cutinases, and lipases, are induced in the presence of Impranil (Zhang et al., 2022). Polydispersity index (PDI), defined as Mw/Mn (where Mw = weight average molecular weight and Mn = number average molecular weight) increased due to biocatalytic reaction (Table 1). The enzymes taking part in PU hydrolysis preferentially broke down the smaller carbon chains of PU having exo-activity (i.e., cleaving from the ends), as implied by the minor Mw reduction. Nevertheless, the enzymatic degradation of PU powder was not so extensive for mass loss to occur. This is in accordance with previous findings whereby the enzymatic treatment of polyether PU results in minor mass loss, probably because of material recalcitrance under tested conditions [24,59].

Table 1. Molecular weight and number (Mw, Mn) and polydispersity index of PU material after *A. mediterranei* ISP5501 whole-cell biocatalysis.

	Mn (g mol^{-1})	Mw (g mol^{-1})	PDI [a]
Initial material	$86,583 \pm 50$	$146,755 \pm 10$	1.69 ± 0.01
Control culture	$83,621 \pm 39$	$144,835 \pm 2772$	1.73 ± 0.06
Whole cells (PU)	$80,500 \pm 781$	$145,065 \pm 202$	1.80 ± 0.01
Whole cells (PU + Impranil)	$74,933 \pm 279$	$146,159 \pm 1219$	1.95 ± 0.02

[a] PDI = polydispersity index.

The enzymes responsible for PU hydrolysis are proteases/amidases and esterases, while several reports mention ureases as potential enzymes for poly(ether urea) PU breakdown [60,61]. Interestingly, none of the aforementioned activities were detected in the *A. mediterranei* culture supernatant, suggesting that the responsible enzymes can be possibly membrane-bound, similar to bacterial strains, such as *C. acidovorans* and *Pseudomonas capeferrum,* and a fungal *Penicillium* strain, which have been reported to possess membrane-bound esterases showing high hydrolytic activity against PU [62–64]. For this reason, *A. mediterranei* ISP5501 was grown in liquid cultures supplemented with Impranil, and the extracellular and/or intracellular protein fractions were utilized for PU degradation. As shown in Table 2, the intracellular fraction decreased Mn by $10.6 \pm 0.3\%$, while in the case of fraction mixture the corresponding decrease was slightly lower. It is noteworthy that the extracellular fraction caused imperceptible PU degradation, implying that the intracellular fraction, which is also enriched with membrane proteins released after cell lysis, possesses all the necessary enzymes for urethane bond cleavage. The esterase

activity reached 19.9 mU mL^{-1} in the intracellular fraction (pNPB assay), whereas no such activity was detected extracellularly. The proteolytic activity (azocasein assay) was not detected in any of the fractions, although this alone does not provide enough evidence for ruling out protease activity, as protease/amidase products were detected using whole cells and PU-7 as substrate (Figure 4).

Table 2. Molecular weight and number (*Mw*, *Mn*) and polydispersity index of PU material after treatment with extracellular and intracellular protein fractions of *A. mediterranei* ISP5501.

	Mn (g mol^{-1})	*Mw* (g mol^{-1})	PDI [a]
Initial material	86,583 ± 50	146,755 ± 10	1.69 ± 0.01
Control (Tris buffer)	88,382 ± 760	147,263 ± 1263	1.67 ± 0.05
Extracellular fraction	84,824 ± 186	148,841 ± 1133	1.75 ± 0.02
Intracellular fraction	77,364 ± 274	146,227 ± 248	1.89 ± 0.01
Mixed fraction	78,170 ± 28	146,603 ± 249	1.88 ± 0.01

[a] PDI = polydispersity index.

To further correlate PU degradation with specific enzymatic activities, the genome of *A. mediterranei* was sequenced and investigated. The *A. mediterranei* ISP5501 genome consists of a 10198110 bp long scaffold, which is in accordance with reference genomes including Gene bank accession NC_022116.1 and is 99.4% complete based on BUSCO analysis. The predicted proteome of ISP5501 consists of 9386 proteins. We classified these proteins into their respective protein families based on the functional annotations of PGAP and InterProScan and we focused on families associated with PU depolymerization. These families include esterases, ureases, proteases, amidases, and other α/β hydrolases (Table 3), the largest of which are proteases in all subcellular locations and in total. Based on the enzyme assays performed, the enzymes predicted as membrane-bound are of great interest. In the nine membrane esterases found, there are three lipases, while three membrane amidohydrolases and six α/β hydrolases were also indicated. Next, we searched for members of these enzyme families in the *A. mediterranei* RefSeq genomes and did not observe any noticeable difference between the ISP5501 and the other genomes regarding these enzyme families (Table S4).

Table 3. PU depolymerization-associated enzyme families in the predicted proteome of *A. mediterranei* ISP5501.

Enzyme Family	Intracellular	Membrane-Bound	Extracellular
Amidases	70	3	14
Esterases	88	9	64
Other α/β hydrolases	123	6	17
Proteases	199	68	120
Ureases	9	0	0

The BLAST searches using known PU-active enzymes as templates identified six possible PU-active enzymes in the ISP5501 proteome. One of them is the extracellular AML cutinase, further corroborating that this enzyme could be responsible for urethane bond cleaving. Two extracellular esterases were also identified as homologous to the PET and PU-active triacylglycerol lipase from *Thermomonospora curvata* (Tcur_1278), an enzyme with hydrolytic activity against poly(ε-caprolactone) [65]. Both esterases (pgaptmp_003900, pgaptmp_004139) have a carbohydrate-binding ricin B lectin domain and have a protein sequence identity of 54.5% and 45.6% with Tcur_1278, respectively. Using the LED HMM profiles, these three candidates were classified as members of family 49, superfamily 1. This family contains PETase-like homologs that consist of the core α/β-hydrolase domain without additional structural modules like lids [66]. We also identified three intracellular carboxylesterases homologous to the polyurethane esterase from *Delftia acidovorans* (PudA) with a protein sequence identity range of 31.0–36.5%. Recently a cutinase from *A. mediterranei* AML (UniProt No- A0A0H3DES9) was investigated for its plastic-degrading

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potential. The enzyme couldn't degrade PLA or PET; however, it could degrade PCL and poly(1,4-butylene succinate) [67]. The search of *A. mediterranei* ISP5501 genome for known etherases yielded intracellular etherase pgaptmp_009080 N-acetylmuramic acid 6-phosphate etherase, which is not likely to be involved in PU degradation due to the specific mechanism of activity on the lactyl side chain N-acetylmuramic acid 6-phosphate.

The antiSMASH analysis of the ISP5501 genome indeed detected the rifamycin biosynthetic gene cluster encoding five type I polyketide synthases that constitute the core biosynthetic genes [68]. The genome of ISP5501 also encodes a putative polyhydroxyalkanoate (PHA) PhaC polymerase (pgaptmp_006620), the key enzyme involved in PHA biosynthesis [69], which is in line with all previous observations that this taxon has a metabolic network both for extensive utilization of various carbon sources and also for effective funneling of metabolic intermediates into the secondary antibiotic synthesis process. Therefore, one can envisage upcycling of polymeric materials into compounds of high value, such as antibiotics.

3. Conclusions

Plastic pollution is a pressing environmental problem the scale of which is still largely unknown. PUs have been identified as one of the most toxic types of plastic and, due to a lack of efficient recycling strategies, present an ongoing issue. In this study, we synthesized eight PU model compounds representing partial hydrolysis products and screening molecules for the identification of novel PU-degrading biocatalysts. When tested on lung fibroblast cells, *A. fischeri* and *C. elegans*, PU degradation products proved more toxic and ecotoxic than PU polymers themselves, a fact that needs to be taken into account when assessing potential PU waste management strategies. The model compound PU-5 proved useful for bond-specific screening, differentiating between ester and urethane bond hydrolysis and aiding in the discovery of urethane bond-specific biocatalysts. Additionally, a novel urethane bond-degrading bacterium, *A. mediterranei* ISP5501, was identified using the PU-7 model compound. *A. mediterranei* ISP5501 is capable of degrading both ester- and ether-based Pus, a highly sought-after feature for PU depolymerization. Given the fact PUs are a heterogenous group of polymers, the use of robust biocatalysts capable of degrading different types of PUs is essential for developing efficient PU degradation processes as a first step to achieve their effective and sustainable conversion into valuable compounds.

4. Materials and Methods

4.1. Reagents

2,4-Toluene diisocyanate, phenyl isocyanate, adipic acid, 2,4-TDA, thiazole orange dye, and all other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. PU polymer Impranil DNL SD (Impranil) was obtained from Covestro (Leverkusen, Germany) and PU polymer Laripur LPR7560 was purchased from Coim group (Milano, Italy).

4.2. PU Model Compounds Synthesis

PU models based on phenyl isocyanate and toluene diisocyanate were synthesized (Figure 1b) using the previously published procedure of reactions of hydroxyalkyl esters with phenyl isocyanate [35] with some modifications. All model compounds were isolated and purified using chromatographic separation methods, and purified compounds were characterized using NMR (Varian/Agilent NMR 400 MHz (^{1}H at 400 MHz, ^{13}C at 100 MHz), Palo Alto, Santa Clara, CA, USA). Samples were dissolved in three different deuterated solvents (CDCl3, CD3OD3, and DMSO-d6). Chemical shifts (δ) are expressed in ppm and coupling constant (J) in Hz. PU-1 and PU-7 were previously known compounds, but not so well structurally characterized [32,33]. For all compounds, detailed preparation procedures and full spectra assignation can be found in the supporting information (Figures S1–S16).

4.3. Ecotoxicity Assessment of PU Model Compounds

4.3.1. Cytotoxicity Evaluation (MTT Assay)

PU-1 to PU-8 along with Impranil, adipic acid, and 2,4-TDA were assessed for their ability to inhibit the proliferation of human lung fibroblast cell line (MRC-5; American Type Culture Collection (ATCC)) by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide). Pre-grown (24 h) cell monolayers (1×10^4 cells per well) in RPMI 1640 medium supplemented with 100 µg mL^{-1} streptomycin, 100 U mL^{-1} penicillin, and 10% (*v/v*) fetal bovine serum (FBS) containing the tested compounds at concentrations ranging from 12.5 to 100 µg mL^{-1} were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C for 48 h. The cell viability (extent of MTT reduction) was measured spectrophotometrically at 540 nm using a plate reader (Epoch 2000, BioTek, Winooski, VT, USA), and the cell survival was expressed as a percentage of the control (untreated cells). Cytotoxicity was expressed as the concentration of the compound inhibiting cell growth by 50% (IC_{50}).

4.3.2. Aliivibrio Fischeri Toxicity Tests

All stock solutions (50 mg mL^{-1}) were dissolved in DMSO. Stock solutions were diluted in 2% NaCl up to 500 µg mL^{-1}. PU-3, PU-5, and PU-8 formed a suspension when mixed with NaCl; therefore, all of the 500 µg mL^{-1} sample solutions were briefly centrifuged for 30 s at 10,000 rpm prior to application. The supernatants were transferred to a 1.5 mL tube and used for the preparation of serial dilutions by diluting each starting concentration by 50% (500–7.81 µg mL^{-1}). Also, the medium for freeze-dried bacteria and reference substance was prepared according to ISO 11348-3 standard. The medium was prepared in a volumetric flask (500 mL) by adding 20 g L^{-1} NaCl, 2.03 g L^{-1} $MgCl_2 \times 6 H_2O$, 0.3 g L^{-1} KCl and deionized water up to 500 mL. $K_2Cr_2O_7$ (105.8 mg L^{-1} prepared in 2% NaCl) was used as a reference substance. DMSO (0.6% prepared in 2% NaCl) was used as control. All solutions were stored at 4 °C until use.

The inhibitory effect on the light emission of *A. fischeri* was determined with BioFix® Lumi-10 (Macherey-Nagel GmbH & Co. KG, Duren, Germany) according to ISO 11348 standard. Freeze-dried bacteria (*A. fischeri* NRRL B-11177, Macherey-Nagel GmbH & Co. KG, Duren, Germany) were firstly reconstituted and stored at 4 °C for 10 min using reconstitution solution and additionally stored at 4 °C for 10 min after adding medium for freeze-dried bacteria. Bacteria were incubated at 15 °C with 1 mL of 2% NaCl with different concentrations of PU model substrates and Impranil. The bioluminescence was monitored after 15 min and 30 min of incubation with the test solution.

4.3.3. In Vivo Toxicity on Caenorhabditis Elegans

Synchronized worms (L4 stage) were suspended in a medium containing 95% M9 buffer (3.0 g of KH_2PO_4, 6.0 g of Na_2HPO_4, 5.0 g of NaCl, and 1 mL of 1 M $MgSO_4 \times 7 H_2O$ in 1 L of water), 5% LB broth (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 10 g L^{-1} NaCl), and 10 µg mL^{-1} of cholesterol. The experiment was carried out in 96-well flat-bottomed microtiter plates (Sarstedt, Nümbrecht, Germany) in the final volume of 100 µL per well. Suspension of nematodes (25 µL containing 25–35 nematodes) was transferred to the wells of a 96-well microtiter plate, where 50 µL of the medium was previously added. Next, 25 µL of a solvent control (DMSO) or 25 µL of a concentrated solution was added to the test wells. The final concentrations of the compounds were 500, 100, 50, 25, and 10 µg mL^{-1}. Subsequently, the plates were incubated at 25 °C for 2 days. The fraction of dead worms was determined after 48 h by counting the number of dead worms and the total number of worms in each well, using a stereomicroscope (SMZ143-N2GG, Motic, Wetzlar, Germany). As a negative control experiment, nematodes were exposed to the medium containing 1% (*v/v*) DMSO.

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4.4. Recombinant Proteins and Enzymatic Degradation of PU Model Substrates

Recombinantly expressed enzymes were a cutinase from *Fusarium oxysporum* (FoCut5a) [70], a cutinase from *Humicola insolens* (HiC) [71], an outer membrane esterase from *Comamonas acidovorans* TB-35 (DaPUase) [72], and a PET hydrolase from *Ideonella sakaiensis* (IsPETase) [73]. The expression vector pET-22b(+) (Novagen, St. Louis, MO, USA) was used for FoCut5a, DaPUase, and IsPETase expression, while pET-26b(+) (Novagen, St. Louis, MO, USA) was used for HiC. When cell growth reached OD_{600} 0.6–0.8, protein expression was induced by the addition of 0.2 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG), and cultures were further incubated for 20 h at 16 °C. After induction, the cultures were centrifuged at $4000 \times g$ for 15 min at 4 °C and the cell pellet was resuspended in 20 mL of 50 mM Tris-HCl buffer containing 300 mM NaCl (pH 8.0). Subsequently, cells were disrupted using an ultrasonic processor (VC 600, Sonics and Materials, Newtown, CT, USA) applying 4 cycles of 60 s sonication (50% Duty Cycle), at 40% amplitude. After disruption, the cell-free extract was collected by centrifugation at $10,000 \times g$ for 20 min at 4 °C and the supernatant was filtered through a 0.45 μm filter. Proteins were purified by immobilized metal-ion affinity chromatography (IMAC). The purity of the isolated enzyme was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and protein concentration was determined by measuring the absorbance at 280 nm, based on the calculated molar extinction coefficient. Fractions containing the purified enzyme were dialyzed overnight at 4 °C against a 20 mM Tris–HCl buffer (pH 8.0).

The enzymatic hydrolysis of the PU-5 model substrate was performed using recombinant enzymes (FoCut5a, HiC, DaPUase, and IsPETase), and commercial protease preparations (protease from *Bacillus licheniformis* (BacProt) (EC 232-752-2) and *Streptomyces griseus* (StrepProt) (EC232-909-5), both purchased from Sigma-Aldrich (St. Louis, MO, USA)). Enzymatic reactions containing 10 mg mL^{-1} of PU substrate and 0.5 μM of each enzyme were performed in 1 mL phosphate buffer pH 7.5, in an Eppendorf Thermomixer Comfort (Eppendorf, Hamburg, Germany) at 30 °C or 50 °C depending on the temperature optimum of the enzyme and 1200 rpm for 24 h. Prior to analysis, 500 μL of methanol was added to the reaction mixture. Afterward, every sample was vortexed and centrifuged at $5000 \times g$ at 10 °C. The reaction products were analyzed by HPLC on an Agilent 1260 Infinity II instrument (Agilent Technologies, Santa Clara, CA, USA) using a C-18 reverse-phase Nucleosil®100-5 (Macherey-Nagel, Düren, Germany) under isocratic conditions using a mobile phase consisting of 39.5% methanol, 59.5% water, and 1% triethylamine at a flow rate of 0.8 mL min^{-1} at 25 °C for 30 min. PU model substrates and degradation products were detected using an Agilent 1260 Infinity II UV Variable Wavelength Detector (G7114B, Agilent Technologies, Santa Clara, CA, USA) at 278 nm.

4.5. Screening and Identification of Microorganisms

An in-house bacterial collection consisting of bacteria isolated from various environmental sites and strains obtained from the Agricultural Research Service (ARS) Culture Collection (Peoria, IL, USA) were screened for their PU-degrading potential using Mineral Salt Medium (15 g L^{-1} agar, 9 g L^{-1} Na$_2$HPO$_4$ × 12 H$_2$O, 1.5 g L^{-1} KH$_2$PO$_4$, 1 g L^{-1} NH$_4$Cl, 0.2 g L^{-1} MgSO$_4$ × 7H$_2$O, 0.2 g L^{-1} CaCl$_2$ × 2H$_2$O, 0.1% trace elements solution, 0.025% N-Z amine) agar plates supplemented with 6 g L^{-1} of Impranil as carbon source. Cultures were incubated for 3–4 weeks at 30 °C. The formation of clearing zones around microorganisms was considered a positive result. *Amycolatopsis mediterranei* ISP5501 strain was stained with thiazole orange after growth on solid media. Briefly, cells were scraped into sterile PBS and diluted to approximately 1×10^6 cells mL^{-1}, washed twice with PBS, fixed with paraformaldehyde solution (4%, *v/v*), and stained with 10 μM of thiazole orange in PBS at 25 °C for 20 min in the dark. Cells were visualized using a fluorescence microscope (Olympus BX51, Applied Imaging Corp., San Jose, CA, USA), under 60 and $100\times$ magnification.

Selected trains were identified by 16S rRNA gene sequencing. The 16S region was amplified via PCR (FastGene TAQ PCR Kit, Nippon Genetics, Düren, Germany) using standard 1496R and 27F primers. Amplicons were sequenced by Macrogen Europe BV (Amsterdam, The Netherlands). Sequences were analyzed using MEGA (Molecular Evolutionary Genetics Analysis) [74] software and the microorganisms were identified using BLAST.

4.6. Whole-Cell Biocatalytic Reactions and Mass Spectrometry Analysis of Degradation Products

Strains used to assess PU model substrate degradation were grown in diluted LB (50%, v/v) with the addition of Impranil (final concentration 0.4%, v/v) at 30 °C and 180 rpm for 72 h. Cells were harvested by centrifugation at $5000 \times g$ and 4 °C for 15 min and washed twice with MSM medium. A total of 100 µL of cell suspension was added to 3 mL MSM medium supplemented with PU model substrates (final concentration 1 g L^{-1}) and the whole-cell reaction was incubated for 3 days at 30 °C and 180 rpm.

Reaction products of whole-cell biocatalytic reactions were analyzed using UHPLC-MS/MS. Samples were centrifuged at $12,000 \times g$ for 15 min and methanol (to a final concentration of 40%) was added. Next, the samples were filtered through a 0.22 µm PTFE filter. A library of predicted ester and urethane bond degradation products ions was constructed for MS screening (Table S2). The analysis was performed on Agilent 1290 Infinity UHPLC with a 6460 Triple Quad MS detector (Agilent Technologies, Santa Clara, CA, USA). For HPLC analysis, a modified method for monitoring 2,4-TDA [75] was used. An amount of 5 µL of the samples were injected and passed through Agilent Zorbax Eclipse Plus C18 column (Agilent Technologies, Santa Clara, CA, USA) (2.1 × 50 mm, 1.8 µm) at a flow rate of 0.4 mL min^{-1} at 25 °C. Mobile phases were A: water with 1% formic acid; and B: acetonitrile with 1% formic acid with a gradient: 0–1 min 40–95% B; 2.5 min 95% B. Electrospray source was operated in positive ion mode with the following common parameters: nitrogen drying gas temperature 300 °C, nitrogen sheath gas temperature 300 °C, nitrogen drying gas flow 10 L min^{-1}, nitrogen sheath gas flow 7.5 L min^{-1}, nebulizer pressure 45 psi, capillary voltage 3500 V and nozzle voltage 500 V. Spectra were acquired and analyzed using Agilent Technologies MassHunter software (Version 10.0).

4.7. Degradation of PU Powder Material

LB medium supplemented with 0.4% (v/v) of Impranil (5 mL) was inoculated with *A. mediterranei* ISP5501 strain and incubated at 30 °C on orbital shaker 180 rpm for 48 h. This culture (1 mL) was used for the inoculation of 100 mL half-strength LB supplemented with 0.4% (v/v) of Impranil. Cultures were incubated at 30 °C and 180 rpm for 72 h and subsequently centrifuged at $5000 \times g$ for 10 min at 4 °C. The supernatant (extracellular fraction) was collected, filter sterilized, and dialyzed against PBS buffer overnight, while the cell pellet of each culture was resuspended in 5 mL PBS buffer. The cells were disrupted using the ultrasonic processor VC 600 (Sonics and Materials, Newtown, CT, USA) applying 4 cycles of 60 s sonication (50% Duty Cycle), at 40% amplitude. After sonication, the disrupted cells were centrifuged at $10,000 \times g$ for 20 min at 4 °C, and the supernatant was filtered through a 0.45 µm filter (intracellular fraction). Total protein mix (mixed fraction) was obtained by mixing the extracellular and intracellular fractions at a ratio of 3:1 (v/v).

Enzyme reactions were performed in an Eppendorf Thermomixer Comfort (Eppendorf, Hamburg, Germany) by incubating 10 mg of polyether PU with 0.26 mg of the extracellular, intracellular, or mixed fraction in 1 mL of 100 mM phosphate buffer (pH 7), at 30 °C for 72 h. After 72 h, PU powder was isolated by centrifugation, and washed with 2% (w/v) SDS solution, followed by a double rinse with ultrapure water. Finally, the powder was again isolated by centrifugation and freeze-dried under a vacuum before its properties were determined. In control samples, PU powder was treated in the same manner, but each of the protein fractions has been previously boiled for 15 min.

Polyether PU (LPR7560, Laripur) in the form of pellets was cryo-milled in a Pulverisette 14 (Fritsch Corp., Idar-Oberstein, Germany), resulting in particle diameter smaller than 500 µm before it was used as a substrate. PU powder was used as substrate with

A. mediterranei ISP5501 whole cells, whereby 100 mg (0.1% w/v) of PU powder was added to cultures, while in some cases 0.4% (v/v) Impranil was also supplemented in the medium. ISP5501 liquid cultures were incubated for 72 h under the aforementioned conditions and subsequently centrifuged at $1000 \times g$ for 10 min at 4 °C, allowing the PU powder to precipitate. After discarding the supernatant, the powder was washed and isolated as mentioned before. Abiotic controls (without bacterial cells) were set up for validating experiment results.

PU material characterization was performed using differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA), as previously described [76]. The determination of the molecular masses of virgin and treated PU powder was performed with gel permeation chromatography (GPC) using two PLgel MIXED-D 5 μm columns (300 × 7.5 mm) in Agilent 1260 Infinity II instrument (Agilent Technologies, Santa Clara, CA, USA), as described previously [76].

4.8. Assessment of A. mediterranei ISP5501 Esterase and Proteinase Activities

The esterase activity of *A. mediterranei* ISP5501 was determined in each protein fraction using *p*-nitrophenyl butyrate (pNPB) as a substrate. The activity assay for each of the fractions was performed using pNPB at 1 mM concentration in 0.1 M phosphate-citrate buffer at pH 6. Reactions were initiated by adding 20 μL of each of the intracellular or extracellular fractions to 230 μL of the substrate and the release of *p*-nitrophenol was monitored by measuring absorbance at 410 nm in a SpectraMax-250 microplate reader (Molecular Devices, Sunnyvale, CA, USA) equipped with SoftMaxPro software (version 1.1, Molecular Devices, Sunnyvale, CA, USA) set at 35 °C. Proteolytic activity was determined using azocasein as substrate after modifying the protocol of Samal et al. [77]. In specific, proteolytic activity was estimated after mixing 25 μL of the enzyme with 0.4 mg azocasein and 175 μL of 0.1 M Tris-HCl buffer pH 8. The reactions were incubated for 20 min at 40 °C. After incubation, 200 μL of 0.1 M trichloroacetic acid (TCA) was added to the reaction mixture, and afterward, the reactions were centrifuged at $3000 \times g$ for 2 min. Then, 200 μL of the supernatant was removed and mixed with 200 μL of 0.5 M NaOH. The absorbance of the final mixture was measured at 440 nm in a SpectraMax-250 microplate reader (Molecular Devices, Sunnyvale, CA, USA) equipped with SoftMaxPro software (version 1.1, Molecular Devices, Sunnyvale, CA, USA). Urease activity was determined after mixing 500 mM urea and 0.002% phenol red in 10 mM K_2HPO_4 solution pH 6.2. Reactions were initiated by adding 25 μL of sample in 225 μL of the reaction mixture. The increase in the absorbance at 570 nm was recorded using a SpectraMax-250 microplate reader [78,79]. Apart from protein fractions, whole-cell samples (full cultures) and culture supernatants were also assayed for esterase, protease, or urease activity. Protein concentration was estimated according to Lowry et al. using BSA as standard solutions [80].

4.9. A. mediterranei ISP5501 Whole-Genome Sequencing, Genome Assembly, and Annotation

A 350-bp insert size library was prepared and sequenced in paired-end mode (read length, 150 bp) by Novogene Europe on a NovaSeq 6000 (Illumina, San Diego, CA, USA) instrument and a total of 5,908,038 paired reads were generated. Raw reads were preprocessed with TrimGalore v0.6.5 and cutadapt v2.9 [81]. The Illumina adapter sequences were removed (with a stringency of 3), bases with a quality score less than 10 were trimmed, and reads smaller than 100 bases or with no pair were discarded. De novo genome assembly was performed with Spades v3.13.0 [82] and was further scaffolded and gaps filled through an improvement pipeline. Genome completeness was assessed with BUSCO v5.1.2 using the Actinobacteria phylum single-copy orthologs from OrthoDB v10 [83].

Gene prediction and functional annotation were performed with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP, release 2022-04-14) [84]. The genome was searched for secondary metabolite biosynthetic gene clusters with antiSMASH v.6.1.1 [85]. Predicted proteins were mapped to KEGG pathways using BlastKOALA [86] and their subcellular localization was predicted with the GP4 pipeline for gram-positive bacteria [87]. Proteins

predicted as extracellular or of unknown localization with GP[4] were further searched for transmembrane proteins with DeepTMHMM [88] since GP[4] cannot predict the *Actinobacteria* outer membrane proteins. The annotated genome assembly has been deposited in GenBank under accession number CP100416.

The predicted proteome of ISP5501 was searched for homologs of PU-active enzymes. All available sequences from biochemically characterized PU-active enzymes were downloaded from PAZy [22] and were used as templates for BLAST searches. The alignments were filtered for protein sequence identity > 30% and for >50% alignment coverage of both the template and the target sequence. Lastly, hidden Markov model profiles of alpha/beta-hydrolase families that contain PU-active enzymes were downloaded from the Lipase Engineering Database (LED) [89] and used to classify the enzymes found with BLAST. Next, we searched for broader enzyme families related to PU hydrolysis. These families include amidases, esterases, proteases, ureases, and other α/β hydrolases. Proteins were assigned to enzyme families with InterProScan v5.56-89.0 and PGAP. All 7 available *A. mediterranei* proteomes from RefSeq were used for comparison with ISP5501 genome.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/catal13020278/s1, Details of synthetic procedures and NMR spectra (Figures S1–S16); PU-5 degradation products after enzymatic treatment (Figure S17); *A. mediterranei* ISP5501 visualized under a fluorescent microscope (Figure S18); solubility of PU model compounds in a selection of common organic solvents (Table S1); list of predicted PU-7 degradation products (Table S2); identification of 22 Impranil-degrading bacterial strains by 16S sequencing (Table S3); PU depolymerization associated enzyme families in *A. mediterranei* genomes (Table S4).

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References

1.　Liu, J.; He, J.; Xue, R.; Xu, B.; Qian, X.; Xin, F.; Blank, L.M.; Zhou, J.; Wei, R.; Dong, W. Biodegradation and up-cycling of polyurethanes: Progress, challenges, and prospects. *Biotechnol. Adv.* **2021**, *48*, 107730. [CrossRef] [PubMed]
2.　Mordorinteligence. Polyurethane Market—Growth, Trends, COVID-19 Impact, and Forecasts (2022–2027). 2021. Available online: https://www.reportlinker.com/p06316155/Polyurethane-Market-Growth-Trends-COVID-19-Impact-and-Forecasts.html?utm_source=GNW (accessed on 28 September 2022).
3.　Zhang, Y.-Q.; Lykaki, M.; Markiewicz, M.; Alrajoula, M.T.; Kraas, C.; Stolte, S. Environmental contamination by microplastics originating from textiles: Emission, transport, fate and toxicity. *J. Haz. Mat.* **2022**, *430*, 128453. [CrossRef] [PubMed]
4.　Liang, C.; Gracida-Alvarez, U.R.; Gallant, E.T.; Gillis, P.A.; Marques, Y.A.; Abramo, G.P.; Hawkins, T.R.; Dunn, J.B. Material flows of polyurethane in the United States. *Environ. Sci. Technol.* **2021**, *55*, 14215–14224. [CrossRef]
5.　Esperanza, M.; Font, R.; GarcíA, A. Toxic byproducts from the combustion of varnish wastes based on polyurethane in a laboratory furnace. *J. Haz. Mat.* **2000**, *77*, 107–121. [CrossRef]
6.　Gadhave, R.V.; Srivastava, S.; Mahanwar, P.A.; Gadekar, P.T. Recycling and disposal methods for polyurethane wastes: A review. *Open J. Polymer Chem.* **2019**, *9*, 39–51. [CrossRef]

7. Zimmermann, L.; Dierkes, G.; Ternes, T.A.; VöLker, C.; Wagner, M. Benchmarking the in vitro toxicity and chemical composition of plastic consumer products. *Environ. Sci. Technol.* **2019**, *53*, 11467–11477. [CrossRef] [PubMed]
8. Yuan, Z.; Nag, R.; Cummins, E. Ranking of potential hazards from microplastics polymers in the marine environment. *J. Haz. Mat.* **2022**, *429*, 128399. [CrossRef]
9. Oz, K.; Merav, B.; Sara, S.; Yael, D. Volatile organic compound emissions from polyurethane mattresses under variable environmental conditions. *Environ. Sci. Technol.* **2019**, *53*, 9171–9180. [CrossRef]
10. Garrido, M.A.; Gerecke, A.C.; Heeb, N.; Font, R.; Conesa, J.A. Isocyanate emissions from pyrolysis of mattresses containing polyurethane foam. *Chemosphere* **2017**, *168*, 667–675. [CrossRef]
11. Luft, A.; BröDer, K.; Kunkel, U.; Schulz, M.; Dietrich, C.; Baier, R.; Heininger, P.; Ternes, T.A. Nontarget analysis via LC-QTOF-MS to assess the release of organic substances from polyurethane coating. *Environ. Sci. Technol.* **2017**, *51*, 9979–9988. [CrossRef]
12. Ibarra, J.C.; Uscátegui, Y.L.; Arévalo, F.R.; Díaz, L.E.; Valero, M.F. Influence of diisocyanate chemical structure on the properties, degradation and cytotoxicity of polyurethanes obtained from castor oil. *J. Biomat. Tissue Eng.* **2018**, *8*, 279–289. [CrossRef]
13. Simón, D.; Borreguero, A.; De Lucas, A.; Rodríguez, J. Recycling of polyurethanes from laboratory to industry, a journey towards the sustainability. *Waste Manag.* **2018**, *76*, 147–171. [CrossRef] [PubMed]
14. Marson, A.; Masiero, M.; Modesti, M.; Scipioni, A.; Manzardo, A. Life Cycle Assessment of polyurethane foams from polyols obtained through chemical recycling. *ACS Omega* **2021**, *6*, 1718–1724. [CrossRef]
15. Tournier, V.; Topham, C.; Gilles, A.; David, B.; Folgoas, C.; Moya-Leclair, E.; Kamionka, E.; Desrousseaux, M.-L.; Texier, H.; Gavalda, S. An engineered PET depolymerase to break down and recycle plastic bottles. *Nature* **2020**, *580*, 216–219. [CrossRef]
16. Jin, X.; Dong, J.; Guo, X.; Ding, M.; Bao, R.; Luo, Y. Current advances in polyurethane biodegradation. *Polym. Int.* **2022**, *71*, 1384–1392. [CrossRef]
17. Mahajan, N.; Gupta, P. New insights into the microbial degradation of polyurethanes. *RSC Adv.* **2015**, *5*, 41839–41854. [CrossRef]
18. Skleničková, K.; Abbrent, S.; Halecký, M.; Kočí, V.; Beneš, H. Biodegradability and ecotoxicity of polyurethane foams: A review. *Crit. Rev. Environ. Sci. Technol.* **2020**, *52*, 157–202. [CrossRef]
19. Magnin, A.; Entzmann, L.; Pollet, E.; Avérous, L. Breakthrough in polyurethane bio-recycling: An efficient laccase-mediated system for the degradation of different types of polyurethanes. *Waste Manag.* **2021**, *132*, 23–30. [CrossRef]
20. Nakajima-Kambe, T.; Onuma, F.; Akutsu, Y.; Nakahara, T. Determination of the polyester polyurethane breakdown products and distribution of the polyurethane degrading enzyme of *Comamonas acidovorans* strain TB-35. *J. Ferment. Bioeng.* **1997**, *83*, 456–460. [CrossRef]
21. PAZy—The Plastics-Active Enzymes Database. Available online: https://pazy.eu/doku.php (accessed on 22 July 2022).
22. Buchholz, P.; Zhang, H.; Perez-Garcia, P.; Nover, L.-L.; Chow, J.; Streit, W.R.; Pleiss, J. Plastics degradation by hydrolytic enzymes: The Plastics-Active Enzymes Database-PAZy. *Proteins* **2021**, *90*, 1443–1456. [CrossRef]
23. Magnin, A.; Pollet, E.; Phalip, V.; Avérous, L. Evaluation of biological degradation of polyurethanes. *Biotechnol. Advan.* **2020**, *39*, 107457. [CrossRef]
24. Magnin, A.; Pollet, E.; Perrin, R.; Ullmann, C.; Persillon, C.; Phalip, V.; Avérous, L. Enzymatic recycling of thermoplastic polyurethanes: Synergistic effect of an esterase and an amidase and recovery of building blocks. *Waste Manag.* **2019**, *85*, 141–150. [CrossRef]
25. Liu, J.; Liu, J.; Xu, B.; Xu, A.; Cao, S.; Wei, R.; Zhou, J.; Jiang, M.; Dong, W. Biodegradation of polyether-polyurethane foam in yellow mealworms (*Tenebrio molitor*) and effects on the gut microbiome. *Chemosphere* **2022**, *304*, 135263. [CrossRef]
26. Chow, J.; Perez-Garcia, P.; Dierkes, R.; Streit, W.R. Microbial enzymes will offer limited solutions to the global plastic pollution crisis. *Microb. Biotechnol.* **2023**, *16*, 195–217. [CrossRef] [PubMed]
27. Zhang, K.; Hu, J.; Yang, S.; Xu, W.; Wang, Z.; Zhuang, P.; Grossart, H.-P.; Luo, Z. Biodegradation of polyester polyurethane by the marine fungus *Cladosporium halotolerans* 6UPA1. *J. Haz. Mat.* **2022**, *437*, 129406. [CrossRef]
28. Howard, G.; Vicknair, J.; Mackie, R. Sensitive plate assay for screening and detection of bacterial polyurethanase activity. *Lett. Appl. Microbiol.* **2001**, *32*, 211–214. [CrossRef]
29. Biffinger, J.C.; Barlow, D.E.; Cockrell, A.L.; Cusick, K.D.; Hervey, W.J.; Fitzgerald, L.A.; Nadeau, L.J.; Hung, C.S.; Crookes-Goodson, W.J.; Russell, J.N., Jr. The applicability of Impranil® DLN for gauging the biodegradation of polyurethanes. *Polym. Degrad. Stabil.* **2015**, *120*, 178–185. [CrossRef]
30. Islam, S.; Apitius, L.; Jakob, F.; Schwaneberg, U. Targeting microplastic particles in the void of diluted suspensions. *Environ. Int.* **2019**, *123*, 428–435. [CrossRef] [PubMed]
31. Gamerith, C.; Acero, E.H.; Pellis, A.; Ortner, A.; Vielnascher, R.; Luschnig, D.; Zartl, B.; Haernvall, K.; Zitzenbacher, S.; Strohmeier, G. Improving enzymatic polyurethane hydrolysis by tuning enzyme sorption. *Polym. Degrad. Stabil.* **2016**, *132*, 69–77. [CrossRef]
32. Nishio, A.; Mochizuki, A.; Sugiyama, J.I.; Takeuchi, K.; Asai, M.; Yonetake, K.; Ueda, M. Synthesis and characterization of ordered polyurethanes from nonsymmetric diisocyanate and ethylene glycol. *J. Polym. Sci. A Polym. Chem.* **2000**, *38*, 2106–2114. [CrossRef]
33. Hikita, T.; Mochizuki, A.; Sugiyama, J.-I.; Takeuchi, K.; Asai, M.; Ueda, M. Head-to-tail regularity of polyurethanes from p-isocyanatobenzyl isocyanate and ethylene glycol by a distannoxane catalyst. *Polym. J.* **2001**, *33*, 547–553. [CrossRef]
34. Chambers, J.; Reese, C.B. The thermal decomposition of some tolylene bis-carbamates. *Brit. Polym. J.* **1977**, *9*, 41–46. [CrossRef]
35. Lubczak, R.; Lubczak, J. Reactions of hydroxyalkyl esters with phenyl isocyanate. *J. Appl. Polym. Sci.* **2005**, *96*, 1357–1367. [CrossRef]

36. Utomo, R.N.C.; Li, W.-J.; Tiso, T.; Eberlein, C.; Doeker, M.; Heipieper, H.J.; Jupke, A.; Wierckx, N.; Blank, L.M. Defined microbial mixed culture for utilization of polyurethane monomers. *ACS Sust. Chem. Eng.* **2020**, *8*, 17466–17474. [CrossRef]

37. Mishra, S.; Pang, S.; Zhang, W.; Lin, Z.; Bhatt, P.; Chen, S. Insights into the microbial degradation and biochemical mechanisms of carbamates. *Chemosphere* **2021**, *279*, 130500. [CrossRef]

38. Amato-Lourenço, L.F.; Carvalho-Oliveira, R.; Júnior, G.R.; Dos Santos Galvão, L.; Ando, R.A.; Mauad, T. Presence of airborne microplastics in human lung tissue. *J. Haz. Mat.* **2021**, *416*, 126124. [CrossRef]

39. Neale, P.A.; Antony, A.; Bartkow, M.E.; Farre, M.J.; Heitz, A.; Kristiana, I.; Tang, J.Y.; Escher, B.I. Bioanalytical assessment of the formation of disinfection byproducts in a drinking water treatment plant. *Environ. Sci. Technol.* **2012**, *46*, 10317–10325. [CrossRef]

40. Ventura, S.P.; De Morais, P.; Coelho, J.A.; Sintra, T.; Coutinho, J.A.; Afonso, C.A. Evaluating the toxicity of biomass derived platform chemicals. *Green Chem.* **2016**, *18*, 4733–4742. [CrossRef]

41. Dodard, S.; Renoux, A.; Hawari, J.; Ampleman, G.; Thiboutot, S.; Sunahara, G. Ecotoxicity characterization of dinitrotoluenes and some of their reduced metabolites. *Chemosphere* **1999**, *38*, 2071–2079. [CrossRef]

42. Šepič, E.; Bricelj, M.; Leskovšek, H. Toxicity of fluoranthene and its biodegradation metabolites to aquatic organisms. *Chemosphere* **2003**, *52*, 1125–1133. [CrossRef]

43. Zimmermann, L.; Göttlich, S.; Oehlmann, J.; Wagner, M.; Völker, C. What are the drivers of microplastic toxicity? Comparing the toxicity of plastic chemicals and particles to Daphnia magna. *Environ. Pollut.* **2020**, *267*, 115392. [CrossRef] [PubMed]

44. Wittkowski, P.; Marx-Stoelting, P.; Violet, N.; Fetz, V.; Schwarz, F.; Oelgeschläger, M.; Schönfelder, G.; Vogl, S. Caenorhabditis elegans as a promising alternative model for environmental chemical mixture effect assessment—A comparative study. *Environ. Sci. Technol.* **2019**, *53*, 12725–12733. [CrossRef]

45. Judy, J.D.; Williams, M.; Gregg, A.; Oliver, D.; Kumar, A.; Kookana, R.; Kirby, J.K. Microplastics in municipal mixed-waste organic outputs induce minimal short to long-term toxicity in key terrestrial biota. *Environ. Pollut.* **2019**, *252*, 522–531. [CrossRef]

46. Mcqueen, C.A.; Williams, G.M. Review of the genotoxicity and carcinogenicity of 4, 4′-methylene-dianiline and 4, 4′-methylene-bis-2-chloroaniline. *Mutat. Res./Rev. Gen. Toxicol.* **1990**, *239*, 133–142. [CrossRef] [PubMed]

47. Cregut, M.; Bedas, M.; Durand, M.-J.; Thouand, G. New insights into polyurethane biodegradation and realistic prospects for the development of a sustainable waste recycling process. *Biotechnol. Adv.* **2013**, *31*, 1634–1647. [CrossRef] [PubMed]

48. Diefenbach, X.W.; Farasat, I.; Guetschow, E.D.; Welch, C.J.; Kennedy, R.T.; Sun, S.; Moore, J.C. Enabling biocatalysis by high-throughput protein engineering using droplet microfluidics coupled to mass spectrometry. *ACS Omega* **2018**, *3*, 1498–1508. [CrossRef]

49. Djapovic, M.; Milivojevic, D.; Ilic-Tomic, T.; Lješević, M.; Nikolaivits, E.; Topakas, E.; Maslak, V.; Nikodinovic-Runic, J. Synthesis and characterization of polyethylene terephthalate (PET) precursors and potential degradation products: Toxicity study and application in discovery of novel PETases. *Chemosphere* **2021**, *275*, 130005. [CrossRef]

50. Yue, W.; Yin, C.-F.; Sun, L.; Zhang, J.; Xu, Y.; Zhou, N.-Y. Biodegradation of bisphenol-A polycarbonate plastic by *Pseudoxanthomonas* sp. strain NyZ600. *J. Haz. Mat.* **2021**, *416*, 125775. [CrossRef]

51. Labow, R.S.; Meek, E.; Santerre, J.P. The biodegradation of poly (urethane) s by the esterolytic activity of serine proteases and oxidative enzyme systems. *J. Biomat. Sci.* **1999**, *10*, 699–713. [CrossRef]

52. Gololobov, M.Y.; Morozova, I.P.; Vojushina, T.L.; Timokhina, E.A.; Stepanov, V.M. Subtilisin from Bacillus subtilis strain 72. The influence of substrate structure, temperature and pH on catalytic properties. *Biochim. Biophys. Acta* **1992**, *1118*, 267–276. [CrossRef]

53. Spasic, J.; Mandic, M.; Djokic, L.; Nikodinovic-Runic, J. *Streptomyces* spp. in the biocatalysis toolbox. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 3513–3536. [CrossRef] [PubMed]

54. Molitor, R.; Bollinger, A.; Kubicki, S.; Loeschcke, A.; Jaeger, K.E.; Thies, S. Agar plate-based screening methods for the identification of polyester hydrolysis by Pseudomonas species. *Microb. Biotechnol.* **2020**, *13*, 274–284. [CrossRef]

55. Singhvi, N.; Singh, P.; Prakash, O.; Gupta, V.; Lal, S.; Bechthold, A.; Singh, Y.; Singh, R.K.; Lal, R. Differential mass spectrometry-based proteome analyses unveil major regulatory hubs in rifamycin B production in *Amycolatopsis mediterranei*. *J. Proteom.* **2021**, *239*, 104168. [CrossRef]

56. Pranamuda, H.; Tokiwa, Y. Degradation of poly (L-lactide) by strains belonging to genus Amycolatopsis. *Biotechnol. Lett.* **1999**, *21*, 901–905. [CrossRef]

57. Gambarini, V.; Pantos, O.; Kingsbury, J.M.; Weaver, L.; Handley, K.M.; Lear, G. Phylogenetic distribution of plastic-degrading microorganisms. *Msystems* **2021**, *6*, e01112–e01120. [CrossRef] [PubMed]

58. Joo, S.; Cho, I.J.; Seo, H.; Son, H.F.; Sagong, H.-Y.; Shin, T.J.; Choi, S.Y.; Lee, S.Y.; Kim, K.-J. Structural insight into molecular mechanism of poly (ethylene terephthalate) degradation. *Nat. Comm.* **2018**, *9*, 382. [CrossRef]

59. Christenson, E.M.; Patel, S.; Anderson, J.M.; Hiltner, A. Enzymatic degradation of poly (ether urethane) and poly (carbonate urethane) by cholesterol esterase. *Biomaterials* **2006**, *27*, 3920–3926. [CrossRef]

60. Loredo-Treviño, A.; Gutiérrez-Sánchez, G.; Rodríguez-Herrera, R.; Aguilar, C.N. Microbial enzymes involved in polyurethane biodegradation: A review. *J. Polym. Environ.* **2012**, *20*, 258–265. [CrossRef]

61. Wei, R.; Zimmermann, W. Microbial enzymes for the recycling of recalcitrant petroleum-based plastics: How far are we? *Microb. Biotechnol.* **2017**, *10*, 1308–1322. [CrossRef]

62. Puiggené, Ò.; Espinosa, M.J.C.; Schlosser, D.; Thies, S.; Jehmlich, N.; Kappelmeyer, U.; Schreiber, S.; Wibberg, D.; Kalinowski, J.; Harms, H. Extracellular degradation of a polyurethane oligomer involving outer membrane vesicles and further insights on the degradation of 2, 4-diaminotoluene in *Pseudomonas capeferrum* TDA1. *Sci. Rep.* **2022**, *12*, 1–12. [CrossRef]

63. Shah, Z.; Krumholz, L.; Aktas, D.F.; Hasan, F.; Khattak, M.; Shah, A.A. Degradation of polyester polyurethane by a newly isolated soil bacterium, *Bacillus subtilis* strain MZA-75. *Biodegradation* **2013**, *24*, 865–877. [CrossRef] [PubMed]
64. Magnin, A.; Hoornaert, L.; Pollet, E.; Laurichesse, S.; Phalip, V.; Avérous, L. Isolation and characterization of different promising fungi for biological waste management of polyurethanes. *Microb. Biotechnol.* **2019**, *12*, 544–555. [CrossRef]
65. Wei, R.; Oeser, T.; Then, J.; Kühn, N.; Barth, M.; Schmidt, J.; Zimmermann, W. Functional characterization and structural modeling of synthetic polyester-degrading hydrolases from *Thermomonospora curvata*. *AMB Express* **2014**, *4*, 44. [CrossRef]
66. Bauer, T.L.; Buchholz, P.C.; Pleiss, J. The modular structure of α/β-hydrolases. *FEBS J.* **2020**, *287*, 1035–1053. [CrossRef] [PubMed]
67. Tan, Y.; Henehan, G.T.; Kinsella, G.K.; Ryan, B.J. An extracellular lipase from Amycolatopsis mediterannei is a cutinase with plastic degrading activity. *Comp. Struct. Biotechnol. J.* **2021**, *19*, 869–879. [CrossRef] [PubMed]
68. Schupp, T.; Toupet, C.; Engel, N.; Goff, S. Cloning and sequence analysis of the putative rifamycin polyketide synthase gene cluster from Amycolatopsis mediterranei. *FEMS Microb. Lett.* **1998**, *159*, 201–267. [CrossRef]
69. Chek, M.F.; Hiroe, A.; Hakoshima, T.; Sudesh, K.; Taguchi, S. PHA synthase (PhaC): Interpreting the functions of bioplastic-producing enzyme from a structural perspective. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 1131–1141. [CrossRef]
70. Dimarogona, M.; Nikolaivits, E.; Kanelli, M.; Christakopoulos, P.; Sandgren, M.; Topakas, E. Structural and functional studies of a *Fusarium oxysporum* cutinase with polyethylene terephthalate modification potential. *Biochim. Biophys. Acta-Gen. Subj.* **2015**, *1850*, 2308–2317. [CrossRef]
71. Ronkvist, Å.M.; Xie, W.; Lu, W.; Gross, R.A. Cutinase-catalyzed hydrolysis of poly (ethylene terephthalate). *Macromolecules* **2009**, *42*, 5128–5138. [CrossRef]
72. Shigeno-Akutsu, Y.; Nakajima-Kambe, T.; Nomura, N.; Nakahara, T. Purification and properties of culture-broth-secreted esterase from the polyurethane degrader *Comamonas acidovorans* TB-35. *J. Biosci. Bioeng.* **1999**, *88*, 484–487. [CrossRef]
73. Yoshida, S.; Hiraga, K.; Takehana, T.; Taniguchi, I.; Yamaji, H.; Maeda, Y.; Toyohara, K.; Miyamoto, K.; Kimura, Y.; Oda, K. A bacterium that degrades and assimilates poly (ethylene terephthalate). *Science* **2016**, *351*, 1196–1199. [CrossRef] [PubMed]
74. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molec. Biol. Evol.* **2018**, *35*, 1547. [CrossRef]
75. Espinosa, M.; Blanco, A.; Schmidgall, T.; Atanasoff-Kardjalieff, A.; Kappelmeyer, U.; Tischler, D.; Pieper, D.; Heipieper, H.; Eberlein, C. Toward biorecycling: Isolation of a soil bacterium that grows on a polyurethane oligomer and monomer. *Front. Microbiol.* **2020**, *11*, 404. [CrossRef]
76. Nikolaivits, E.; Taxeidis, G.; Gkountela, C.; Vouyiouka, S.; Maslak, V.; Nikodinovic-Runic, J.; Topakas, E. A polyesterase from the Antarctic bacterium Moraxella sp. degrades highly crystalline synthetic polymers. *J. Haz. Mat.* **2022**, *434*, 128900. [CrossRef]
77. Samal, B.B.; Karan, B.; Stabinsky, Y. Stability of two novel serine proteinases in commercial laundry detergent formulations. *Biotechnol. Bioeng.* **1990**, *35*, 650–652. [CrossRef]
78. Goldie, J.; Veldhuyzen Van Zanten, S.; Jalali, S.; Hollingsworth, J.; Riddell, R.; Richardson, H.; Hunt, R. Optimization of a medium for the rapid urease test for detection of *Campylobacter pylori* in gastric antral biopsies. *J. Clin. Microbiol.* **1989**, *27*, 2080–2082. [CrossRef] [PubMed]
79. Tanaka, T.; Kawase, M.; Tani, S. Urease inhibitory activity of simple α, β-unsaturated ketones. *Life Sci* **2003**, *73*, 2985–2990. [CrossRef] [PubMed]
80. Lowry, O.; Rosebrough, N.; Farr, A.; Randall, R. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275. [CrossRef] [PubMed]
81. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* **2011**, *17*, 10–12. [CrossRef]
82. Bankevich, A.; Nurk, S.; Antipov, D.; Gurevich, A.A.; Dvorkin, M.; Kulikov, A.S.; Lesin, V.M.; Nikolenko, S.I.; Pham, S.; Prjibelski, A.D. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J. Comp. Biol.* **2012**, *19*, 455–477. [CrossRef]
83. Manni, M.; Berkeley, M.R.; Seppey, M.; Simão, F.A.; Zdobnov, E.M. BUSCO update: Novel and streamlined workflows along with broader and deeper phylogenetic coverage for scoring of eukaryotic, prokaryotic, and viral genomes. *Molec. Biol. Evol.* **2021**, *38*, 4647–4654. [CrossRef] [PubMed]
84. Li, W.; O'neill, K.R.; Haft, D.H.; Dicuccio, M.; Chetvernin, V.; Badretdin, A.; Coulouris, G.; Chitsaz, F.; Derbyshire, M.K.; Durkin, A.S. RefSeq: Expanding the prokaryotic genome annotation pipeline reach with protein family model curation. *Nucleic Acids Res.* **2021**, *49*, D1020–D1028. [CrossRef]
85. Blin, K.; Shaw, S.; Kloosterman, A.M.; Charlop-Powers, Z.; Van Wezel, G.P.; Medema, M.H.; Weber, T. antiSMASH 6.0: Improving cluster detection and comparison capabilities. *Nucleic Acids Res.* **2021**, *49*, W29–W35. [CrossRef]
86. Kanehisa, M.; Sato, Y.; Morishima, K. BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *J. Mol. Biol.* **2016**, *428*, 726–731. [CrossRef] [PubMed]
87. Grasso, S.; Van Rij, T.; Van Dijl, J.M. GP4: An integrated Gram-positive protein prediction pipeline for subcellular localization mimicking bacterial sorting. *Brief Bioinformat.* **2021**, *22*, bbaa302. [CrossRef] [PubMed]

88. Hallgren, J.; Tsirigos, K.D.; Pedersen, M.D.; Armenteros, J.J.A.; Marcatili, P.; Nielsen, H.; Krogh, A.; Winther, O. DeepTMHMM predicts alpha and beta transmembrane proteins using deep neural networks. *bioRxiv* **2022**. [CrossRef]

89. Biocatnet, The Lipase Engineering Database (LED). Available online: http://www.led.uni-stuttgart.de/ (accessed on 25 July 2022).

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

RESEARCH ARTICLE

MICROBIAL BIOTECHNOLOGY
Open Access

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).

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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
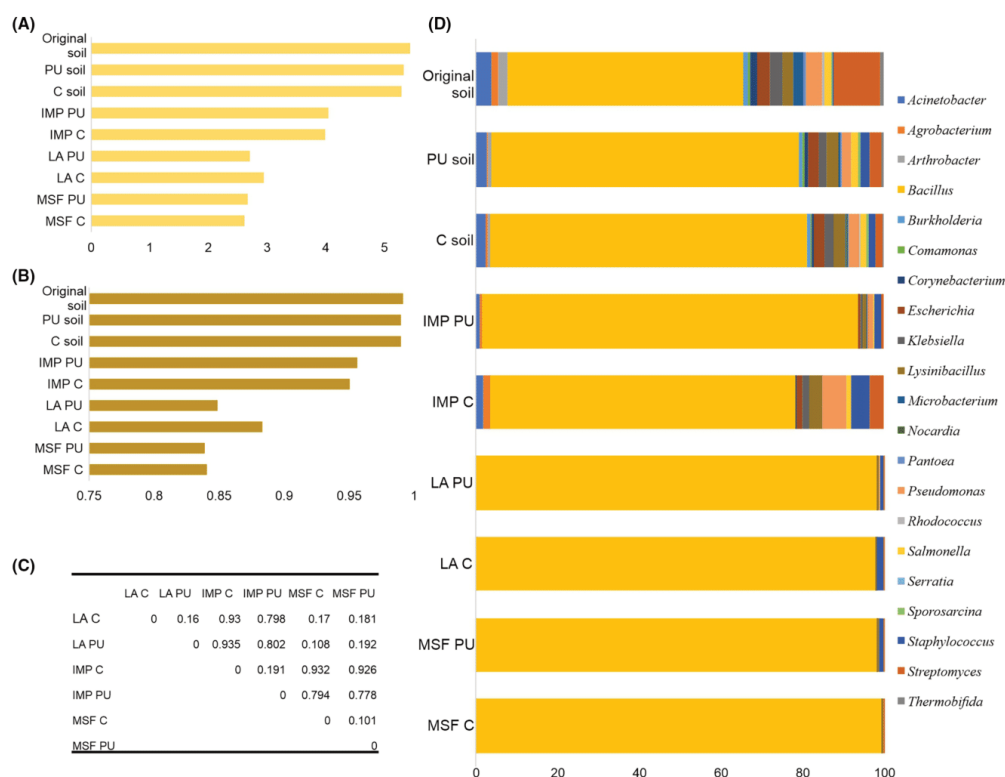

FIGURE 1 Changes in microbial diversity caused by PU enrichment of soil samples. (A) Shannon's diversity index of K, C and PU soil samples; (B) Inverse Simpson's index of K, C and PU soil samples (C) Bray–Curtis dissimilarity index of communities isolated from LA, MSF and IMP plates; (D) Distribution 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) respectively, 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.
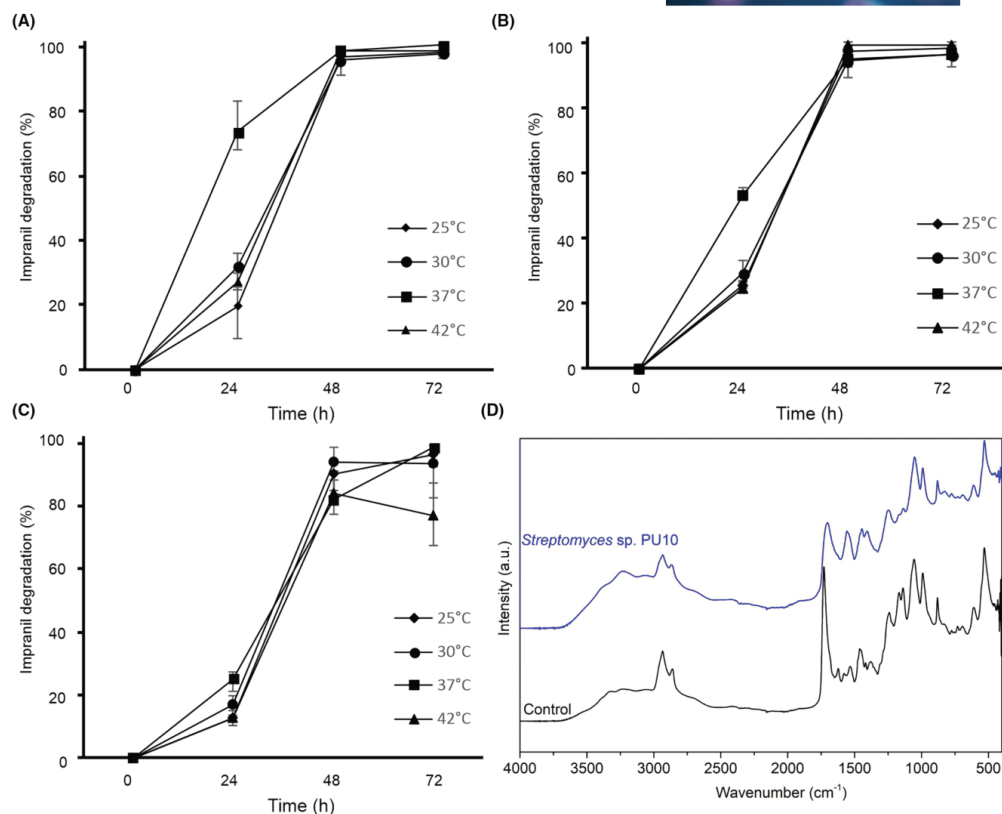
FIGURE 2 Impranil degradation by *Streptomyces* sp. PU10 in liquid culture (performed in triplicate) (A) 0.5% (v/v) of Impranil, (B) 1% (v/v) of Impranil, (C) 1.5% (v/v) of Impranil and (D) FTIR analysis of culture supernatants after 24 h of degradation.

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
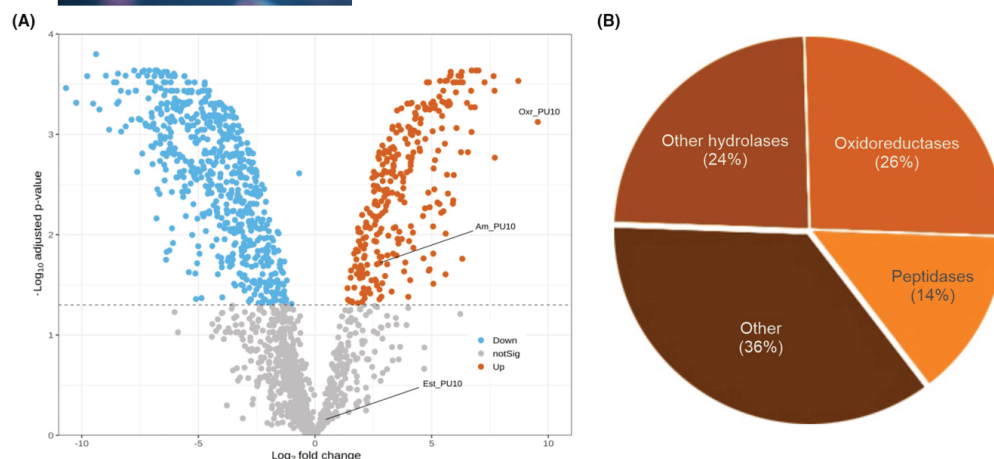
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FIGURE 3 (A) Volcano plot showing the log-fold change and Benjamini–Hochberg adjusted *p*-value of each protein detected and annotated within *Streptomyces* PU10 proteome. Proteins significantly induced in Impranil or glucose are highlighted in orange 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
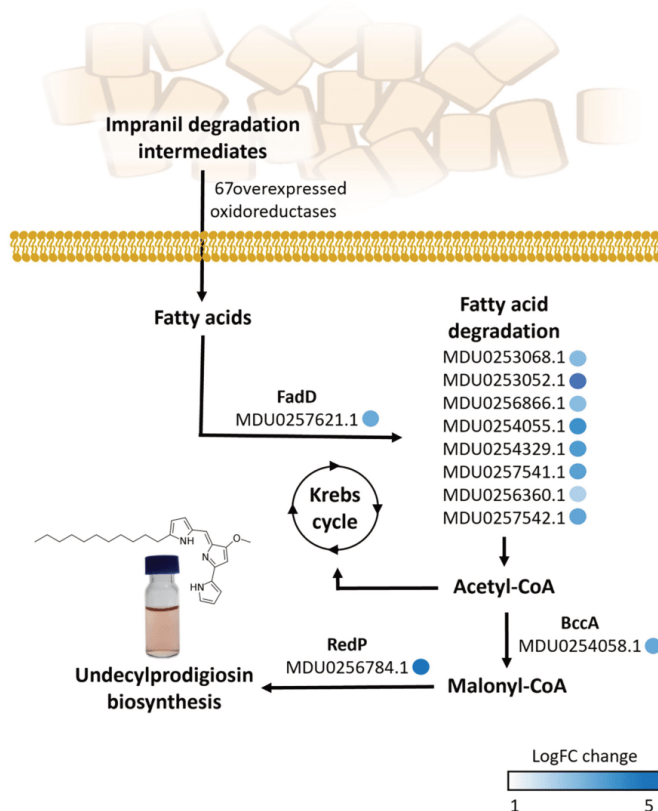
substrates (Hume et al., 2009) and has been linked to effective secondary metabolite production (Banchio & Gramajo, 2002). In addition to MDU0257621.1, two more copies of FadD (MDU0253472.1 and MDU0253870.1) have been detected during growth on Impranil. The resulting acetyl-CoA form fatty acid degradation 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.

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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REFERENCES
Alam, K., Mazumder, A., Sikdar, S., Zhao, Y.-M., Hao, J., Song, C. et al. (2022) Streptomyces: the biofactory of secondary metabolites. *Frontiers in Microbiology*, 13, 968053.

Banchio, C. & Gramajo, H. (2002) A stationary-phase acyl-coenzyme a synthetase of Streptomyces coelicolor A3 (2) is necessary for the normal onset of antibiotic production. *Applied and Environmental Microbiology*, 68, 4240–4246.

Bhavsar, P., Bhave, M. & Webb, H.K. (2023) Effective multi-stage biodegradation of commercial bulk polyurethane by Clonostachys and Purpureocillium spp. *Science Total Environment*, 908, 168329.

Biffinger, J.C., Barlow, D.E., Cockrell, A.L., Cusick, K.D., Hervey, W.J., Fitzgerald, L.A. et al. (2015) The applicability of Impranil® DLN for gauging the biodegradation of polyurethanes. *Polymer Degradation and Stability*, 120, 178–185.

Blin, K., Shaw, S., Augustijn, H.E., Reitz, Z.L., Biermann, F., Alanjary, M. et al. (2023) antiSMASH 7.0: new and improved predictions for detection, regulation, chemical structures and visualisation. *Nucleic Acids Research*, 51, gkad344.

Branson, Y., Söltl, S., Buchmann, C., Wei, R., Schaffert, L., Badenhorst, C.P. et al. (2023) Urethanases for the enzymatic hydrolysis of low molecular weight carbamates and the recycling of polyurethanes. *Angewandte Chemie*, 62, e202216220.

Breitwieser, F.P., Baker, D.N. & Salzberg, S.L. (2018) KrakenUniq: confident and fast metagenomics classification using unique k-mer counts. *Genome Biology*, 19, 1–10.

Breitwieser, F.P. & Salzberg, S.L. (2020) Pavian: interactive analysis of metagenomics data for microbiome studies and pathogen identification. *Bioinformatics*, 36, 1303–1304.

Bu, D., Luo, H., Huo, P., Wang, Z., Zhang, S., He, Z. et al. (2021) KOBAS-i: intelligent prioritization and exploratory visualization of biological functions for gene enrichment analysis. *Nucleic Acids Research*, 49, W317–W325.

Buchholz, P., Zhang, H., Perez-Garcia, P., Nover, L.-L., Chow, J., Streit, W.R. et al. (2022) Plastics degradation by hydrolytic enzymes: the plastics-active enzymes database-PAZy. *Proteins: Structure, Function, and Bioinformatics*, 90(7), 1443–1456.

Carr, C.M., Keller, M.B., Paul, B., Schubert, S.W., Clausen, K.S., Jensen, K. et al. (2023) Purification and biochemical characterization of SM14est, a PET-hydrolyzing enzyme from the marine sponge-derived *Streptomyces* sp. SM14. *Frontiers in Microbiology*, 14, 1170880.

Chen, C.-C., Dai, L., Ma, L. & Guo, R.-T. (2020) Enzymatic degradation of plant biomass and synthetic polymers. *Nature Reviews Chemistry*, 4, 114–126.

Cuebas-Irizarry, M.F. & Grunden, A.M. (2023) *Streptomyces* spp. as biocatalyst sources in pulp and paper and textile industries: biodegradation, bioconversion and valorization of waste. *Microbial Biotechnology*, 00, 1–22.

Di Bisceglie, F., Quartinello, F., Vielnascher, R., Guebitz, G.M. & Pellis, A. (2022) Cutinase-catalyzed polyester-polyurethane degradation: elucidation of the hydrolysis mechanism. *Polymers*, 14, 411.

El-Shafei, H.A., Abd El-Nasser, N.H., Kansoh, A.L. & Ali, A.M. (1998) Biodegradation of disposable polyethylene by fungi and *Streptomyces* species. *Polymer Degradation and Stability*, 62, 361–365.

Erickson, E., Gado, J.E., Avilán, L., Bratti, F., Brizendine, R.K., Cox, P.A. et al. (2022) Sourcing thermotolerant poly (ethylene terephthalate) hydrolase scaffolds from natural diversity. *Nature Communications*, 13, 7850.

Gambarini, V., Pantos, O., Kingsbury, J.M., Weaver, L., Handley, K.M. & Lear, G. (2021) Phylogenetic distribution of plastic-degrading microorganisms. *Msystems*, 6, e01112-20.

Gambarini, V., Pantos, O., Kingsbury, J.M., Weaver, L., Handley, K.M. & Lear, G. (2022) PlasticDB: a database of microorganisms and proteins linked to plastic biodegradation. *Database*, 2022, baac008.

Gangoiti, J., Santos, M., Prieto, M.A., de la Mata, I., Serra, J.L. & Llama, M.J. (2012) Characterization of a novel subgroup of extracellular medium-chain-length polyhydroxyalkanoate depolymerases from actinobacteria. *Applied and Environmental Microbiology*, 78, 7229–7237.

Gui, Z., Liu, G., Liu, X., Cai, R., Liu, R. & Sun, C. (2023) A Deep-Sea bacterium is capable of degrading polyurethane. *Microbiology Spectrum*, 11, e00073-23.

Han, Y., Wang, R., Wang, D. & Luan, Y. (2024) Enzymatic degradation of synthetic plastics by hydrolases/oxidoreductases. *International Biodeterioration & Biodegradation*, 189, 105746.

Ho, T.-F., Ma, C.-J., Lu, C.-H., Tsai, Y.-T., Wei, Y.-H., Chang, J.-S. et al. (2007) Undecylprodigiosin selectively induces apoptosis in human breast carcinoma cells independent of p53. *Toxicology and Applied Pharmacology*, 225, 318–328.

Hu, D.X., Withall, D.M., Challis, G.L. & Thomson, R.J. (2016) Structure, chemical synthesis, and biosynthesis of prodiginine natural products. *Chemical Reviews*, 116, 7818–7853.

Hume, A.R., Nikodinovic-Runic, J. & O'Connor, K.E. (2009) FadD from *pseudomonas putida* CA-3 is a true long-chain fatty acyl coenzyme a synthetase that activates phenylalkanoic and alkanoic acids. *Journal of Bacteriology*, 191, 7554–7565.

Jin, X., Dong, J., Guo, X., Ding, M., Bao, R. & Luo, Y. (2022) Current advances in polyurethane biodegradation. *Polymer International*, 71, 1384–1392.

Jones, P., Binns, D., Chang, H.-Y., Fraser, M., Li, W., McAnulla, C. et al. (2014) InterProScan 5: genome-scale protein function classification. *Bioinformatics*, 30, 1236–1240.

Kanehisa, M., Sato, Y. & Morishima, K. (2016) BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *Journal of Molecular Biology*, 428, 726–731.

Kang, T., Lin, J., Yang, L. & Wu, M. (2021) Expression, isolation, and identification of an ethanol-resistant ethyl carbamate-degrading amidase from *Agrobacterium tumefaciens* d3. *Journal of Bioscience and Bioengineering*, 132, 220–225.

Kashiwagi, N., Ogino, C. & Kondo, A. (2017) Production of chemicals and proteins using biomass-derived substrates from a Streptomyces host. *Bioresource Technology*, 245, 1655–1663.

Khruengsai, S., Sripahco, T. & Pripdeevech, P. (2022) Biodegradation of polyester polyurethane by Embarria clematidis. *Frontiers in Microbiology*, 13, 874842.

Klopfenstein, D., Zhang, L., Pedersen, B.S., Ramírez, F., Warwick Vesztrocy, A., Naldi, A. et al. (2018) GOATOOLS: a python library for gene ontology analyses. *Scientific Reports*, 8, 10872.

Laakel, M., Lebrihi, A., Khaoua, S., Schneider, F., Lefebvre, G. & Germain, P. (1994) A link between primary and secondary metabolism: malonyl-CoA formation in *Streptomyces ambofaciens* growing on ammonium ions or valine. *Microbiology*, 140, 1451–1456.

Li, W., O'Neill, K.R., Haft, D.H., DiCuccio, M., Chetvernin, V., Badretdin, A. et al. (2021) RefSeq: expanding the prokaryotic genome annotation pipeline reach with protein family model curation. *Nucleic Acids Research*, 49, D1020–D1028.

Liang, C., Gracida-Alvarez, U.R., Gallant, E.T., Gillis, P.A., Marques, Y.A., Abramo, G.P. et al. (2021) Material flows of polyurethane in the United States. *Environmental Science & Technology*, 55, 14215–14224.

Liao, Z., Zhang, J., Shi, Y., Zhang, Y., Ma, Z., Bechthold, A. et al. (2022) Improvement of rimocidin biosynthesis by increasing supply of precursor malonyl-CoA via over-expression of acetyl-CoA carboxylase in *Streptomyces rimosus* M527. *Current Microbiology*, 79, 174.

Liu, J., He, J., Xue, R., Xu, B., Qian, X., Xin, F. et al. (2021) Biodegradation and up-cycling of polyurethanes: Progress, challenges, and prospects. *Biotechnology Advances*, 48, 107730.

Liu, J., Zeng, Q., Lei, H., Xin, K., Xu, A., Wei, R. et al. (2023) Biodegradation of polyester polyurethane by *Cladosporium* sp. P7: evaluating its degradation capacity and metabolic pathways. *Journal of Hazardous Materials*, 448, 130776.

Liu, X., Ahmad, S., Ma, J., Wang, D. & Tang, J. (2023) Comparative study on the toxic effects of secondary nanoplastics from biodegradable and conventional plastics on *Streptomyces coelicolor* M145. *Journal of Hazardous Materials*, 460, 132343.

Liu, Z., Fang, Z., Zheng, N., Yang, K., Sun, Z., Li, S. et al. (2023) Chemical upcycling of commodity thermoset polyurethane foams towards high-performance 3D photo-printing resins. *Nature Chemistry*, 15, 1–7.

Lu, J., Rincon, N., Wood, D.E., Breitwieser, F.P., Pockrandt, C., Langmead, B. et al. (2022) Metagenome analysis using the kraken software suite. *Nature Protocols*, 17, 2815–2839.

Luft, A., Bröder, K., Kunkel, U., Schulz, M., Dietrich, C., Baier, R. et al. (2017) Nontarget analysis via LC-QTOF-MS to assess

the release of organic substances from polyurethane coating. *Environmental Science & Technology*, 51, 9979–9988.

MacLean, J., Mayanna, S., Benning, L.G., Horn, F., Bartholomäus, A., Wiesner, Y. et al. (2021) The terrestrial plastisphere: diversity and polymer-colonizing potential of plastic-associated microbial communities in soil. *Microorganisms*, 9, 1876.

Magnin, A., Entzmann, L., Pollet, E. & Avérous, L. (2021) Breakthrough in polyurethane bio-recycling: an efficient laccase-mediated system for the degradation of different types of polyurethanes. *Waste Management*, 132, 23–30.

Magnin, A., Hoornaert, L., Pollet, E., Laurichesse, S., Phalip, V. & Avérous, L. (2019) Isolation and characterization of different promising fungi for biological waste management of polyurethanes. *Microbial Biotechnology*, 12, 544–555.

Magnin, A., Pollet, E., Perrin, R., Ullmann, C., Persillon, C., Phalip, V. et al. (2019) Enzymatic recycling of thermoplastic polyurethanes: synergistic effect of an esterase and an amidase and recovery of building blocks. *Waste Management*, 85, 141–150.

Magnin, A., Pollet, E., Phalip, V. & Avérous, L. (2020) Evaluation of biological degradation of polyurethanes. *Biotechnology Advances*, 39, 107457.

Manni, M., Berkeley, M.R., Seppey, M., Simão, F.A. & Zdobnov, E.M. (2021) BUSCO update: novel and streamlined workflows along with broader and deeper phylogenetic coverage for scoring of eukaryotic, prokaryotic, and viral genomes. *Molecular Biology and Evolution*, 38, 4647–4654.

Molitor, R., Bollinger, A., Kubicki, S., Loeschcke, A., Jaeger, K.E. & Thies, S. (2020) Agar plate-based screening methods for the identification of polyester hydrolysis by *Pseudomonas* species. *Microbial Biotechnology*, 13, 274–284.

Morado, E.G., Paterson, M.L., Ivanoff, D.G., Wang, H.-C., Johnson, A., Daniels, D. et al. (2023) End-of-life upcycling of polyurethanes using a room temperature, mechanism-based degradation. *Nature Chemistry*, 15, 569–577.

Narancic, T., Djokic, L., Kenny, S.T., O'Connor, K.E., Radulovic, V., Nikodinovic-Runic, J. et al. (2012) Metabolic versatility of grampositive microbial isolates from contaminated river sediments. *Journal of Hazardous Materials*, 215, 243–251.

Odom, A.R., Faits, T., Castro-Nallar, E., Crandall, K.A. & Johnson, W.E. (2023) Metagenomic profiling pipelines improve taxonomic classification for 16S amplicon sequencing data. *Scientific Reports*, 13, 13957.

Olanrewaju, O.S. & Babalola, O.O. (2019) Streptomyces: implications and interactions in plant growth promotion. *Applied Microbiology and Biotechnology*, 103, 1179–1188.

Oskay, M. (2009) Comparison of *Streptomyces* diversity between agricultural and non-agricultural soils by using various culture media. *Scientific Research and Essay*, 4, 997–1005.

Pantelic, B., Skaro Bogojevic, S., Milivojevic, D., Ilic-Tomic, T., Lončarević, B., Beskoski, V. et al. (2023) Set of small molecule polyurethane (PU) model substrates: Ecotoxicity evaluation and identification of PU degrading biocatalysts. *Catalysts*, 13, 278.

Park, W.J., Hwangbo, M. & Chu, K.-H. (2023) Plastisphere and microorganisms involved in polyurethane biodegradation. *Science Total Environment*, 886, 163932.

Perez-Riverol, Y., Bai, J., Bandla, C., García-Seisdedos, D., Hewapathirana, S., Kamatchinathan, S. et al. (2022) The PRIDE database resources in 2022: a hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Research*, 50, D543–D552.

PlasticsEurope. (2022) Plastics – the Facts 2022 [Online]. https://plasticseurope.org/

Rüthi, J., Bölsterli, D., Pardi-Comensoli, L., Brunner, I. & Frey, B. (2020) The "plastisphere" of biodegradable plastics is characterized by specific microbial taxa of alpine and arctic soils. *Frontiers in Environmental Science*, 8, 562263.

Rüthi, J., Cerri, M., Brunner, I., Stierli, B., Sander, M. & Frey, B. (2023) Discovery of plastic-degrading microbial strains isolated from the alpine and Arctic terrestrial plastisphere. *Frontiers in Microbiology*, 14, 1178474.

Schmidt, J., Wei, R., Oeser, T., Dedavid e Silva, L.A., Breite, D., Schulze, A. et al. (2017) Degradation of polyester polyurethane by bacterial polyester hydrolases. *Polymers*, 9, 65.

Shepherdson, E.M., Baglio, C.R. & Elliot, M.A. (2023) *Streptomyces* behavior and competition in the natural environment. *Current Opinion in Microbiology*, 71, 102257.

Shi, J., Zeng, Y.J., Zhang, B., Shao, F.L., Chen, Y.C., Xu, X. et al. (2019) Comparative genome mining and heterologous expression of an orphan NRPS gene cluster direct the production of ashimides. *Chemical Science*, 10, 3042–3048.

Simón, D., Borreguero, A., De Lucas, A. & Rodríguez, J. (2018) Recycling of polyurethanes from laboratory to industry, a journey towards the sustainability. *Waste Management*, 76, 147–171.

Sonnendecker, C., Oeser, J., Richter, P.K., Hille, P., Zhao, Z., Fischer, C. et al. (2022) Low carbon footprint recycling of post-consumer PET plastic with a metagenomic polyester hydrolase. *ChemSusChem*, 15, e202101062.

Soud, S.A. (2019) Biodegradation of polyethylene LDPE plastic waste using locally isolated Streptomyces sp. *Journal of Pharmaceutical Sciences and Research*, 11, 1333–1339.

Spasic, J., Mandic, M., Djokic, L. & Nikodinovic-Runic, J. (2018) *Streptomyces* spp. in the biocatalysis toolbox. *Applied Microbiology and Biotechnology*, 102, 3513–3536.

Stankovic, N., Radulovic, V., Petkovic, M., Vuckovic, I., Jadranin, M., Vasiljevic, B. et al. (2012) *Streptomyces* sp. JS520 produces exceptionally high quantities of undecylprodigiosin with antibacterial, antioxidative, and UV-protective properties. *Applied Microbiology and Biotechnology*, 96, 1217–1231.

Sulaiman, S., Yamato, S., Kanaya, E., Kim, J.-J., Koga, Y., Takano, K. et al. (2012) Isolation of a novel cutinase homolog with polyethylene terephthalate-degrading activity from leaf-branch compost by using a metagenomic approach. *Applied and Environmental Microbiology*, 78, 1556–1562.

Summers, R.G., Ali, A., Shen, B., Wessel, W.A. & Hutchinson, C.R. (1995) Malonyl-coenzyme a: acyl carrier protein acyltransferase of *Streptomyces glaucescens*: a possible link between fatty acid and polyketide biosynthesis. *The Biochemist*, 34, 9389–9402.

Taxeidis, G., Nikolaivits, E., Siaperas, R., Gkountela, C., Vouyiouka, S., Pantelic, B. et al. (2023) Triggering and identifying the polyurethane and polyethylene-degrading machinery of filamentous fungi secretomes. *Environmental Pollution*, 325, 121460.

Teufel, F., Almagro Armenteros, J.J., Johansen, A.R., Gíslason, M.H., Pihl, S.I., Tsirigos, K.D. et al. (2022) SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nature Biotechnology*, 40, 1023–1025.

Tournier, V., Topham, C., Gilles, A., David, B., Folgoas, C., Moya-Leclair, E. et al. (2020) An engineered PET depolymerase to break down and recycle plastic bottles. *Nature*, 580, 216–219.

Utomo, R.N.C., Li, W.-J., Tiso, T., Eberlein, C., Doeker, M., Heipieper, H.J. et al. (2020) Defined microbial mixed culture for utilization of polyurethane monomers. *ACS Sustainable Chemistry & Engineering*, 8, 17466–17474.

Vargas-Suárez, M., Fernández-Cruz, V. & Loza-Tavera, H. (2019) Biodegradation of polyacrylic and polyester polyurethane coatings by enriched microbial communities. *Applied Microbiology and Biotechnology*, 103, 3225–3236.

Vargas-Suárez, M., Savín-Gámez, A., Domínguez-Malfavón, L., Sánchez-Reyes, A., Quirasco-Baruch, M. & Loza-Tavera, H. (2021) Exploring the polyurethanolytic activity and microbial composition of landfill microbial communities. *Applied Microbiology and Biotechnology*, 105, 7969–7980.

Wagener, S., Völker, T., De Spirt, S., Ernst, H. & Stahl, W. (2012) 3, 3′-Dihydroxyisorenieratene and isorenieratene prevent UV-induced DNA damage in human skin fibroblasts. *Free Radical Biology & Medicine*, 53, 457–463.

Wang, J., Liu, H., Huang, D., Jin, L., Wang, C. & Wen, J. (2017) Comparative proteomic and metabolomic analysis of *Streptomyces tsukubaensis* reveals the metabolic mechanism of FK506 overproduction by feeding soybean oil. *Applied Microbiology and Biotechnology*, 101, 2447–2465.

Wei, R., Oeser, T., Then, J., Kühn, N., Barth, M., Schmidt, J. et al. (2014) Functional characterization and structural modeling of synthetic polyester-degrading hydrolases from *Thermomonospora curvata*. *AMB Express*, 4, 1–10.

Whicher, J.R., Florova, G., Sydor, P.K., Singh, R., Alhamadsheh, M., Challis, G.L. et al. (2011) Structure and function of the RedJ protein, a thioesterase from the prodiginine biosynthetic pathway in *Streptomyces coelicolor*. *The Journal of Biological Chemistry*, 286, 22558–22569.

Yoshida, S., Hiraga, K., Takehana, T., Taniguchi, I., Yamaji, H., Maeda, Y. et al. (2016) A bacterium that degrades and assimilates poly (ethylene terephthalate). *Science*, 351, 1196–1199.

Yuan, Z., Nag, R. & Cummins, E. (2022) Ranking of potential hazards from microplastics polymers in the marine environment. *Journal of Hazardous Materials*, 429, 128399.

Zabala, D., Braña, A.F., Salas, J.A. & Méndez, C. (2016) Increasing antibiotic production yields by favoring the biosynthesis of precursor metabolites glucose-1-phosphate and/or malonyl-CoA in *Streptomyces* producer strains. *The Journal of Antibiotics*, 69, 179–182.

Zhang, H., Dierkes, R.F., Perez-Garcia, P., Costanzi, E., Dittrich, J., Cea, P.A. et al. (2023) The metagenome-derived esterase PET40 is highly promiscuous and hydrolyses polyethylene terephthalate (PET). *The FEBS Journal*, 291, 70–91.

Zhang, K., Hu, J., Yang, S., Xu, W., Wang, Z., Zhuang, P. et al. (2022) Biodegradation of polyester polyurethane by the marine fungus *Cladosporium halotolerans* 6UPA1. *Journal of Hazardous Materials*, 437, 129406.

Zimmermann, L., Dierkes, G., Ternes, T.A., Völker, C. & Wagner, M. (2019) Benchmarking the in vitro toxicity and chemical composition of plastic consumer products. *Environmental Science & Technology*, 53, 11467–11477.

SUPPORTING INFORMATION

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1.4.Upcycling biodegradable PVA/starch film to a bacterial biopigment and biopolymer

Upcycling Biodegradable PVA/Starch Film to a Bacterial Biopigment and Biopolymer

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Abstract: Meeting the challenge of circularity for plastics requires amenability to repurposing post-use, as equivalent or upcycled products. In a compelling advancement, complete circularity for a biodegradable polyvinyl alcohol/thermoplastic starch (PVA/TPS) food packaging film was demonstrated by bioconversion to high-market-value biopigments and polyhydroxybutyrate (PHB) polyesters. The PVA/TPS film mechanical properties (tensile strength (σ_u), 22.2 ± 4.3 MPa; strain at break (ε_u), 325 ± 73%; and Young's modulus (E), 53–250 MPa) compared closely with low-density polyethylene (LDPE) grades used for food packaging. Strong solubility of the PVA/TPS film in water was a pertinent feature, facilitating suitability as a carbon source for bioprocessing and microbial degradation. Biodegradability of the film with greater than 50% weight loss occurred within 30 days of incubation at 37 °C in a model compost. Up to 22% of the PVA/TPS film substrate conversion to biomass was achieved using three bacterial strains, *Ralstonia eutropha* H16 (*Cupriavidus necator* ATCC 17699), *Streptomyces* sp. JS520, and *Bacillus subtilis* ATCC6633. For the first time, production of the valuable biopigment (undecylprodigiosin) by *Streptomyces* sp. JS520 of 5.3 mg/mL and the production of PHB biopolymer at 7.8% of cell dry weight by *Ralstonia eutropha* H16 from this substrate were reported. This low-energy, low-carbon post-use PVA/TPS film upcycling model approach to plastic circularity demonstrates marked progress in the quest for sustainable and circular plastic solutions.

Keywords: biopolymers; thermoplastic starch; mechanical properties; PVA; biodegradation; upcycling; biopigments

1. Introduction

Upcyclable products meet the conditions required for circularity by being indefinitely recyclable, without reduction in value or usability. The cyclical repurposing of plastic resources, as opposed to contributing to recalcitrant waste stockpiles, is essential to achieving a sustainable socio-economic ecosystem. Progressing plastics circularity requires minimizing polluting factors and resource loss while facilitating continuous material repurposing. Circularity and regenerative cycles are fundamental to the Earth's natural ecosystems. In nature, examples of the biodegradation and bioregeneration of processes for natural polymers and end-of-life bio-based materials abound. In applications spanning from medical devices to food packaging, biodegradable blends and their composites are playing an increasing role in mitigating against negative environmental impacts [1]. Active developments are ongoing to overcome biodegradable plastics performance shortcomings such as brittleness, gas-barrier properties, and processability [1,2]. In addressing

the considerable challenge of circularity, it is vital that post-use regeneration routes for these new plastics are identified to secure resource and value continuity throughout the repurposing/revalorization process [3,4].

Starch can be obtained from the waste streams and by-products of multiple renewable food sources, including corn and potatoes [5,6]. Thermoplastic starch (TPS) is a relatively low-cost material that lacks rigidity and mechanical strength [1,7]. Conversion of starch to a thermoplastic material can be achieved by adding small-molecule plasticizers such as glycerol, sorbitol, and urea [8], or by blending with other polymers such as polycaprolactone (PCL), polylactic acid (PLA), or polyvinyl alcohol (PVA and PVOH) [9,10]. A recent advancement in starch modification allowed its application as a water treatment agent [11].

PVA is a water-soluble synthetic polymer with good barrier properties afforded by its polar hydroxyl groups. PVA is highly processable, but brittle, and its biodegradation has been repeatedly demonstrated [12]. When compounded together, PVA and TPS have been shown to form blends with barrier and mechanical parameters suitable for food packaging applications, while also being biodegradable. Cost-competitive PVA/TPS blends have been demonstrated as alternatives to the widely used recalcitrant food packaging barrier layer plastic, ethylene vinyl alcohol (EVOH) [13,14]. The current cost of starch-based packaging films is not cost-competitive compared to petroleum-based plastics; however, starch blends significantly reduce the costs of bio-based biodegradables and, with the rising costs of petroleum, may be competitive in the near future [15]. Potential applications in food packaging and recent advances in the production of starch-based films are high and have been reviewed recently [16]. For comparison, the current cost of PVA/TPS pellets used in this work is between EUR 3/kg and 4/kg, while the cost of PHB is estimated to be between EUR 10/kg and 24/kg which is still multiple times more than petroleum-based plastics such as PET [17]. As the plastics market and value chain move toward increased sustainability and circularity, the cost of biopolymers, including PVA, TPS, and blends thereof, furcate to strongly decrease in cost as production levels greatly increase.

Investigation into the biochemical pathways of PVA degradation started as early as the 1970s. The proposed mechanism of degradation involves two enzymes, both isolated from *Pseudomonas* species. First, an oxidase (a secondary alcohol oxidase [18] or a specific PVA oxidase [19]) leads to the formation of β-hydroxyketone groups, which are later cleaved by the second enzyme, a β-diketone hydrolase [18]. A number of *Pseudomonas* strains have been identified as PVA degraders [20], along with *Bacillus* [21,22] and several fungal yeast strains [12].

TPS has been reported to be completely biodegradable under all environmental conditions [23]. It has been shown that starch films degrade completely in compost at 60 °C within 30–84 days [24,25]. More recently, the degradation of PVA/TPS blend films by two strains isolated from compost has been reported. Strains *Bacillus* sp. DG22 and *Paenibacillus* sp. DG14 were found to degrade the films to a high degree (45–75% depending on the type of blend used) [21]. The degradation of neat PVA films has been reported to be less than 20% [26] with the increased amenability degradation as part of a blend in keeping with literature reports.

Polyhydroxyalkanoates (PHA) are another type of biodegradable and sustainable polymer that possesses properties similar to petroleum-based polymers [27]. PHAs can be obtained by the fermentation of sustainable feedstocks such as food waste [28]. *Ralastonia eutropha* H16 (*Cupriavidus necator* H16) is a well-known producer of the PHA homopolymer polyhydroxybutyrate (PHB) and is able to convert a variety of substrates [29]. In the search for low-cost substrates, PHB-producing bacteria *Azotobacter chroococcum* and *Haloferax mediterranei* were able to produce PHB using starch as the sole carbon source [30]. *R. eutropha* has been shown to efficiently produce PHB from waste potato starch [31]. To the best of our knowledge, no reports on the production of PHB from PVA have been reported. While the upcycling of packaging materials to biodegradable plastics has been attempted previously, obtaining bacterial biopigments from packaging materials is a novel concept. Bacterial pigments have been attracting attention as natural colorants, which are a

safer and more sustainable alternative to synthetic colorants with applications in food and pharma industries [32].

In this work, PVA/TPS films (in a 1:1 ratio), targeted for packaging applications, were produced by extrusion and characterized. The application of this film as a carbon substrate for the production of value-added microbial biopigments compounds such as undecylprodigiosin [33], and biopolymers such as polyhydroxyalkanoates (specifically PHB), was demonstrated. In addition, the biodegradation of this material was assessed in a compost model system at 37 °C under different conditions. In the quest for sustainable and circular plastic solutions, an array of new polymer blends and composites that encompass upcycling capacities are being developed.

2. Materials and Methods

2.1. Materials and Chemicals

Extruded films of 0.01–0.02 mm in thickness were made from 'Biosol' pellets (B000129EP) and were supplied by EcoBioCroatia Ltd. (Zagreb, Croatia). 'Biosol' pellets contain: 50–60% PVA, 30–40% TPS, 10–20% glycerol, and 1–5% coconut oil, according to the manufacturer's specifications.

Inorganic salts (K_2SO_4, $Na_2HPO_4 \times 12H_2O$, KH_2PO_4, NH_4Cl, $MgSO_4 \times 7H_2O$, and $CaCl_2 \times 2H_2O$), PVA (87–90% hydrolyzed, average molecular weight 30,000–70,000), and solvents were purchased from Sigma-Aldrich (Munich, Germany). Microbiological media and components such as yeast extract, tryptone, and casamino acids were sourced from Oxoid (Thermo Fisher Scientific, Cambridge, UK).

2.2. ATR-Infrared Spectroscopy (ATR-FTIR)

The Fourier transform infrared spectroscopy of PVA/starch films before and after degradation was recorded using an IR-Affinity spectrophotometer (Thermo Scientific, NICOLET iS10, Waltham, MA, USA). The spectra were collected in the range from 4000 to 400 cm^{-1} at room temperature with a scan step of 4 cm^{-1}, and 32 scans were carried out in total.

2.3. Differential Scanning Calorimetry and Thermogravimetric Analysis (DSC/TG)

Coupled differential scanning calorimetry (DSC)/thermogravimetric (TG) analysis was performed on a TA Instruments SDT Q600 instrument (TA instruments Ireland, Dublin, Ireland). Analysis of the starting PVA/TPS film was measured over a temperature range from 25 °C to 900 °C, at a heating rate of 10 °C/min, in a nitrogen atmosphere. The weight of each sample was approximately 3 mg.

2.4. Atomic Force Microscopy (AFM) Analysis

The surface morphology was investigated by atomic force microscopy (AFM) with a NanoScope 3D (Veeco, Plainview, NY, USA) microscope operated in contact mode under ambient conditions. Silicon nitride probes with a spring constant of 0.07–0.4 Nm^{-1} were used. Image analysis was carried out using Nanoscope image processing software. 'Top-view' images display the selected image from a top-down perspective, while height information is represented by the color at a given point. 'Surface Plot' images display the selected image with color-coded height information in a two-dimensional perspective.

The surface roughness value (RMS) was calculated as the root-mean-square average of height deviations taken from the mean data plane, as given by Equation (1):

$$\sqrt{\frac{\sum Z_i^2}{n}} = R_q \tag{1}$$

where Z_i is the maximum vertical distance between the highest and the lowest data points in the image.

2.5. Mechanical Properties of Films

The tensile mechanical properties of cast PVA/starch films were evaluated at ambient temperature using a Shimadzu Autograph AGS-X (Kyoto, Japan) servo-hydraulic universal test machine, equipped with a 1 kN load cell. A crosshead speed of 5 mm/min was used for testing and stress was applied until complete fracture of the samples occurred. Five samples in the form of filmstrips (75 mm × 15 mm × 0.01 mm) were used for testing.

Tensile tests were also carried out on extruded PVA/TPS films (in a 1:1 ratio) using an Ametek TA-1 texture analyzer (Berwyn, IL, USA). The machine was equipped with a 20 N load cell and tests were run using a crosshead speed of 5 mm/min. Due to the anisotropic nature of the extruded film, testing was carried out on samples cut in both the extruded (longitudinal) and perpendicular (transverse) directions. The film, with a thickness of ~0.02 mm, was cut using a die with a width of 5 mm and total length of 100 mm, which resulted in a gauge length of 33 mm when placed between the grips. Soft rubber strips were placed between the sample and stainless-steel grips to reduce local stress concentrations in samples and prevent premature grip failure.

For all tensile tests, the average values ± standard deviation of the tensile strength, strain-at-break, and Young's modulus were reported. The Young's modulus values were calculated in the 0–5% strain region, where elastic behavior occurred in all cases.

2.6. Light Fastness

Optical properties of the film samples before and after degradation under different composting conditions were determined by measuring percent transmittance using a UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan). The film samples were cut into rectangular pieces and placed directly in the side of spectrophotometer magnetic cells using air as a reference with a wavelength range of 200–900 nm [34]. Transparency of the films was tested by measuring the percentage transmittance at 260 nm, and the average values of percent transmittance of three samples for each film was presented.

2.7. Study of Films Water-Contact Properties

2.7.1. Swelling Index (*Q* %)

The swelling index, *Q*, was investigated in phosphate-buffered solution (PBS) over 60 min, at room temperature, according to the procedure adopted from Costa et al. [35]. PVA/TPS packaging samples, with dimensions of 50 mm × 15 mm × 0.01 mm, were dried in an oven at 105 °C until a constant mass was reached, after which the samples were placed in vials containing 50 mL of PBS solution. After 60 min, the films were collected, the excess PBS was removed using absorbent paper, and the films were weighed. The increase in mass was presented as swelling index (*Q* %) according to Equation (2):

$$Q\,\% = \frac{m_s - m_0}{m_0} \times 100 \qquad (2)$$

where m_s is the mass of the swollen film and m_0 is the initial mass of the dry film. The experiment was performed in triplicate and presented as an average value ± standard deviation (SD).

2.7.2. Solubility (*ML* %)

The solubility of PVA/TPS commercial material was determined by employing a procedure described by Flores et al. [36] with adaptation as follows: films (50 mm × 15 cm × 0.01 mm) were dried in an oven at 105 °C prior to immersion in 50.0 mL of PBS. The vials were kept with constant stirring at room temperature for 48 h. The solubility index indicated the mass lost (*ML* %) by each sample and was calculated according to Equation (3):

$$ML\,\% = \frac{m_1 - m_0}{m_0} \times 100 \qquad (3)$$

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where m_1 is the final mass of the dried sample after immersion in PBS and m_0 is the starting mass of the sample. Each measurement was performed in triplicate and the results were expressed as the average value $\pm$ standard deviation (SD).

2.7.3. Water Uptake (*WU* %)

Starch and PVA are highly hygroscopic materials. The moisture content of the PVA/TPS films was investigated by conditioning the samples at room temperature (20–25 °C) in desiccators at controlled humidity levels using saturated potassium sulfate solution (98% K_2SO_4 as a humectant). The experiment was performed in triplicate, and the water uptake was tracked over seven days. The water uptake, *WU* %, was calculated according to the following equation:

$$WU \% = \frac{W_t - W_i}{W_i} \qquad (4)$$

where W_t represents the weight after immersion at a predetermined time t at a 98% relative humidity of the films, and W_i refers to the initial weight of dry film [37].

2.8. Upcycling and Biodegradation Assessment

2.8.1. PVA/TPS Film as Substrate for Bacterial Growth

Bacterial strains (*Bacillus subtilis* ATCC6633, *Streptomyces* sp. JS520 [38], and *Ralstonia eutropha* H16 (*Cupriavidus necator* ATCC 17699) [39]) were grown in tryptone soy broth overnight at 30 °C and 180 rpm. These starter cultures were centrifuged (10,000 rpm/20 min/4 °C), washed with 5 mL of PBS, and used for inoculation (10%, v/v).

The films were rinsed with ethanol and dissolved in hot (~90 °C) sterile H_2O (40 g/L). This solution was used as a carbon source. To a Mineral Salt Medium (MSM; 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, and 0.025% N-Z amine) [40], PVA/TPS solution was added to final concentrations of 20 g/L and 10 g/L. In order to test the potential of these microorganisms to utilize PVA as a sole carbon source, MSM was supplemented with filter-sterilized PVA in 5 g/L and 10 g/L final concentrations.

Erlenmeyer flasks (100 mL volume) containing 20 mL of media were inoculated with 2 mL of washed-overnight cultures and further incubated for 14 days at 30 °C/180 rpm. At the end of incubation, cultures were centrifuged (5000 rpm/20 min/4 °C), the bacterial pellet was dried for 3 days at 65 °C, and the weight was determined.

From the *Streptomyces* sp. JS520 culture grown on PVA/TPS films, undecylprodigiosin was extracted and quantified spectrophotometrically as described previously [38]. A wavelength scan from 200 to 800 nm was taken (Spectrophotometer Ultrospec 3300pro, Amersham Biosciences, Amersham, UK) and the absorption maxima at 533 nm were used for calculation.

From the *R. eutropha* H16 culture grown on PVA/TPS films, PHB was quantified by gas chromatography as described previously from the dried biomass, where 8–10 mg of dried cells was methanolized and the PHB monomers were extracted in chloroform [41].

2.8.2. Model-Compost Degradation

Biodegradation of the PVA/TPS films in model compost was carried out at a constant temperature of 37 °C for 30 days using the protocol described by Ponjavic et al. [42] (Figure S1). The model compost consisted of a commercial mixture of raw materials for the cultivation of white button mushroom (80%, w/w) and commercial universal soil for garden and potting (20%, w/w), both from ACS Garden, Belgrade, Serbia. Pieces of film (3×5) cm^2 (weighing ~38 mg) were placed into a Petri dish and buried in compost, and changes in film appearance were compared by taking photographs.

Three types of compost were used: nontreated compost, heat-pretreated (120 °C, 20 min), and bioaugmented compost. The bioaugmented compost was enriched with $\log_{10}$ 4 cells (*R. eutropha* H16, *Streptomyces* sp. JS520, and *B. subtilis* ATCC6633) per gram of compost and mixed thoroughly with a sterile spatula. A fresh aliquot of bacterial cultures

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was added after 2 weeks, and the appropriate amount of sterile water was added every 5 days to ensure constant humidity. Film samples were removed, washed with cold water, air-dried, and photographed on days 6, 10, 20, and 30. Samples were removed on days 10 and 30 for further analysis and weight measurements.

3. Results and Discussion

3.1. Thermal and Mechanical Properties of PVA/TPS Films

Thermal properties of the PVA/TPS film material are presented in Figure 1, where a representative DSC thermogram and TGA curve are shown. DSC analysis indicated one endothermic, melting peak at 203.6 °C, coming from the PVA, which is in agreement with data from the literature for films prepared from PVA/TPS [43], while the heat melt, ΔH_m, was 74.3 J/g. From the TGA/DTG plots, it was evident that the degradation of the film occurred in a few steps, as four characteristic peaks were detected. The first two degradation steps, where less than 20 weight % was lost, occurred between 20 and 180 °C, and represented the evaporation/dehydration, due to the PVA/TPS known high hydrophilicity and tendency to adsorb moisture from the environment.
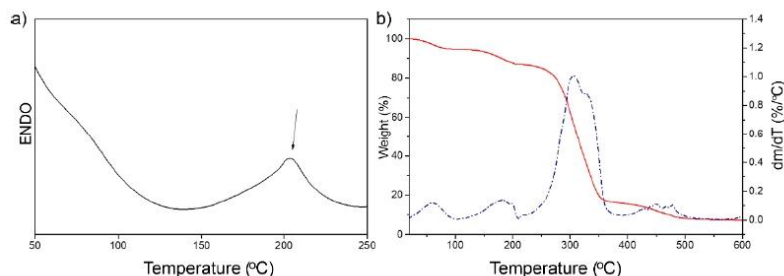
Figure 1. Representative (**a**) DSC thermogram and (**b**) TG/DTGA curves for tested PVA/TPS films.

The main degradation peak at the temperature range from 306 to 332 °C was associated with the degradation of PVA and TPS. The first stage of PVA degradation commenced at 306 °C, while the degradation of TPS commenced at 332 °C. The overlapping of the two main degradation temperatures is due to the compatibility between PVA and starch, where a more thermally stable cyclic hemiacetal in starch improves thermal stability within the PVA/TPS blends [44]. The DTG results indicated that the maximum degradation temperature was at 306 °C when the first degradation step of PVA occurred. This corresponds to the elimination of low-molar-mass carbon-based molecules that are products from the breakage of the polymer carbon-carbon backbone associated with the elimination of hydroxyl groups from dehydration reactions [45]. The second degradation step of PVA occurred at the higher temperature range from 450 to 477 °C and was attributed to the thermal decomposition of high-molar-mass polyenes that are derived during the first stage of degradation [46]. An amount of 90% of weight was lost at 460 °C, and the residue at 500 °C was 8.3 wt%. In the case of TPS, the thermal degradation peak due to decomposition appeared as a 'shoulder' of the PVA main degradation peak at 332 °C, when breaking the starch structure with CO and CO_2 elimination, and the formation of carbonaceous residues occurred [47].

Mechanical properties of the cast PVA/TPS films used for the composting degradation experiments were determined in terms of tensile strength (σ_u), strain at break (ε_u), and Young's modulus (*E*). Representative stress–strain curves are presented in Figure 2 and a summary of the results is given in Table 1.

Figure 2. Mechanical stability of PVA/TPS films.

Table 1. Summary of tensile test results, including tensile strength (σ_u), strain at break (ε_u), and Young's modulus (E).

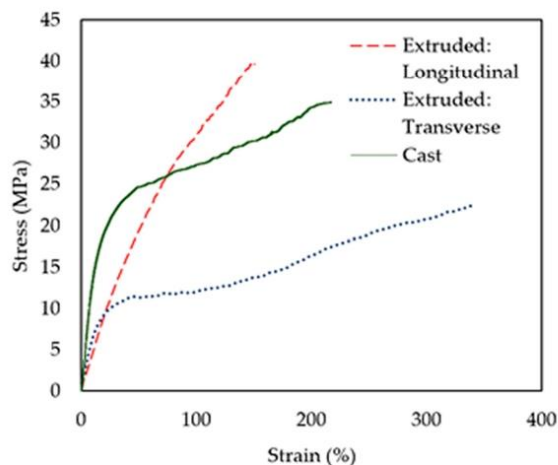|	Cast	Extruded: Longitudinal	Extruded: Transverse
σ_u (MPa)	37.2 ± 2.2	38.6 ± 4.6	22.2 ± 4.3
ε_u (%)	197 ± 70.0	136 ± 17.0	325 ± 73.0
E (GPa)	249.9 ± 94.7	60.3 ± 20.4	52.9 ± 14.1

Within blends, PVA facilitates good mechanical properties by interfacial adhesion between the PVA and different polymers used to prepare PVA blend films [48]. Blending PVA with TPS has the potential to decrease the mechanical properties, including tensile strength and strain at break. The investigated PVA/TPS cast films showed an average strain at break (ε_u) value of 197% and tensile strength (σ_u) of 37.2 MPa, which are higher compared to previously reported PVA/TPS blend films of the same composition (1:1) [49]. The even distribution of TPS strands is one of the most important factors that dictates the mechanical properties of PVA/TPS blend films. High plastic deformation and increased TPS content could promote agglomeration and void formation within the polymer matrix [48], impeding interfacial adhesion between the two components and subsequently decreasing the tensile strength [50]. The high mechanical performance of the tested PVA/TPS films can hence be attributed to the excellent distribution of the TPS polymer chains within the PVA polymer matrix, providing good interfacial compatibility, and the smooth surface of the films, as was observed by both AFM and SEM analysis (PVA/TPS control).

The longitudinal samples demonstrated almost purely elastic behavior with an average tensile strength σ_u of 38.6 MPa (Figure 2). The transverse samples demonstrated very different behavior with a limited elastic region up to around 6–10 MPa, followed by a large plastic area, and failure occurring at an average strength of 22.2 MPa. The elastic regions were similar at earlier stages of testing between the longitudinal (average E of 60.3 MPa) and transverse (average E of 52.9 MPa) cases. However, as elongation continued, the transverse samples deformed plastically, demonstrating high strains-to-break (average ε_u of 325%) compared to the longitudinal samples (average ε_u of 136%). The degree of anisotropy demonstrated from tensile testing is typical for extruded films, due to chain alignment in the extruded (longitudinal) direction during processing.

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All measured mechanical properties of the tested materials are similar to those reported in the literature for PVA/TPS films [49]. The properties compare well with polyethylene films, whose properties are shown for comparison in Table 2. The low modulus and high strength of the PVA/TPS material mean that it is extremely flexible while maintaining the load-bearing capacity required in demanding applications.

Table 2. Range of PVA/TPS mechanical properties compared to those for typical polyethylene film materials (low-density polyethylene (LDPE), high-density polyethylene HDPE, linear low-density polyethylene (LLDPE)) [51], PVA [52], and TPS [53].

Property	PVA/TPS	LDPE	HDPE	LLDPE	PVA	TPS
σ_u (MPa)	22–39	36–57	38–44	36–60	22–30	4–8
ε_u (%)	136–325	300–500	600–860	450–850	99–112	35–100
E (MPa)	53–250	190–520	827–1069	204–275	64–176	116–294

PVA/TPS films indicated a high swelling index, *Q*, after only 60 min of immersion in PBS due to the highly hydrophilic nature of both PVA and starch polymers, while the solubility index, *ML*, was calculated to be 7.5, which meant that after 48 h in PBS, 7.5% of polymer films was dissolved in the medium.

Water uptake was followed by immersion in a saturated solution of potassium sulfate over seven days, and the results are presented in Figure 3. In the starting 48 h of immersion, approximately 40% of moisture was absorbed, while a significant increase in moisture uptake was detected after 72 h of exposure (80%). After 72 h, the water uptake started to decrease, probably due to the dissolution of highly hygroscopic PVA/TPS material. A water uptake of less than 60% was calculated after seven days of exposure.
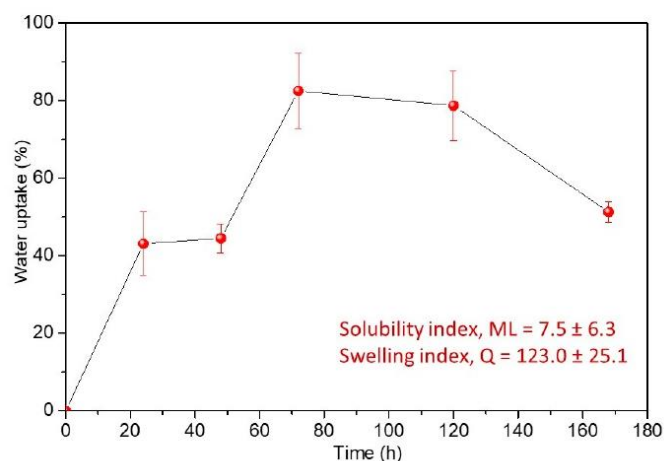
Figure 3. Water uptake of investigated PVA/TPS films over seven days.

The water absorption is a very important property of biopolymers as it directly affects their degradability, and the study of water uptake is essential especially in relation to food packaging applications. Most biodegradable polymers possess a high sensitivity to water absorption. Once the water is absorbed into the polymer, it makes this material suitable for microorganisms to grow, and the fungi and bacteria can utilize the polymer as an energy source [50,54]. Both PVA and starch are highly hydrophilic polymers due to the free –OH groups that form hydrogen bonds with water [8]. However, after the blending of PVA and starch, the decrease in water uptake was observed in comparison to neat PVA and starch as a consequence of hydrogen bonding between PVA and starch, which further affects the

quantity of free –OH groups, causing the decrease in water sensitivity [8,55]. Finally, the investigated PVA/TPS films with a water uptake of more than 80% after 3 days of exposure to moisture appeared as a highly hydrophilic material that would be easily degraded by microorganisms and used as a bacteria energy source.

3.2. Bacterial Upcycling and Biodegradation of PVA/TPS Material

The upcycling of a variety of carbon-rich waste streams including nonoxygenated PE and PP wax into PHA has been achieved [56,57]. Postconsumer PET has been successfully upcycled to PHA and a novel bio-based poly(amide urethane) (bio-PU) recently [58]. In this study, the upcycling of PVA/TPS material to bacterial biopigments and PHB was assessed. PVA/TPS film was cut into strips, comprehensively characterized by various techniques, and used for biotechnological upcycling and biodegradation assessment.

3.2.1. PVA/TPS Films and PVA as Carbon Source for Bacterial Growth

All of the cultures were able to use PVA/TPS films as the sole carbon and energy source in liquid culture (Figure 4). Biomass yields were low to moderate with conversion rates between 8% and 14.5% when 10 g/L of PVA/TPS substrate was used (Table 3). Conversion rates were 1.3–1.8-fold higher when double the amount of substrate was used, with *Streptomyces* sp. JS520 reaching a conversion rate of 22% (Table 3). This strain was able to produce purple biopigment, undecylprodigiosin, under both conditions (Figure 4) to up to 5.3 mg/L of culture (Figure S2).
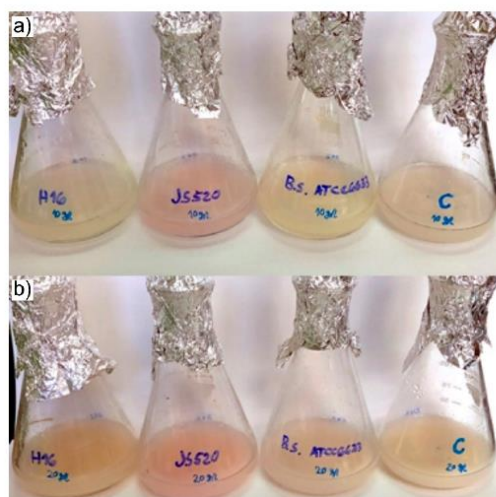
Figure 4. Cultures of *Ralstonia eutropha* H16, *Streptomyces* sp. JS520, *Bacillus subtilis* ATCC6633, and uninoculated control (left to right) in MSM medium with dissolved PVA/TPS material as the sole source of carbon: (**a**) 10 g/L and (**b**) 20 g/L.

The *Streptomyces* JS520 strain was previously reported to produce undecylprodigiosin under optimized conditions of 67 mg/L of culture [38]. Although 12.6-fold lower levels of this biopigment were reached in this study, this is the first report of the upcycling of PVA/TPS polymeric materials to biopigments, and further optimizations of the bioprocess could reach higher titers. Undecylprodigiosin and other pigments of the prodigiosin-family have been shown to exhibit multiple bioactive properties, including anticancer [33,59], and are of high commercial value (approx. EUR 360/mg from Sigma-Aldrich supplier) [60]. Previously, fructose and sucrose were found to stimulate the production of undecylprodigiosin in *Streptomyces* spp. [61], while solid substrates such as wheat bran and rice husk

stimulated its production in solid-state fermentation [62]. Recently, bioprocessed chitinous marine waste was used for the production of pigment from the prodigiosin family [63].

Table 3. Biomass yield (mg dry mass/mL of culture) and conversion rate from bacterial cultures grown in MSM medium containing 10 and 20 g/L of PVA/TPS film.

Strain	MSM + PVA/TPS Film 10 g/L		MSM + PVA/TPS Film 20 g/L	
	Biomass, mg/mL	Conversion, %	Biomass, mg/mL	Conversion, %
Ralstonia eutropha H16	0.80 ± 0.08	8	2.75 ± 0.05	13.7
Streptomyces sp. JS520	1.20 ± 0.09	12	4.40 ± 0.08	22.0
Bacillus subtilis ATCC6633	1.45 ± 0.04	14.5	3.95 ± 0.04	19.7

On the other hand, *R. eutropha* H16, a known PHB producer, showed the least ability to grow on a PVA/TPS carbon source (Table 3) but was also able to accumulate PHB up to 8% of cell dry weight (data not shown). This is considered a moderate to low yield for a biopolymer, but it is the first time that the production of PHB from a PVA/TPS blend has been reported, and, as in the case with undecylprodigiosin, further optimization of the bioprocess would afford higher titers of PHB. The low biomass yields could be due to the fact that wild-type *R. eutropha* H16 lacks gene coding for amylases or glucoamylases, preventing the direct utilization of starch by this strain [64]. Therefore, the obtained biomass and PHB could be due to the utilization of PVA monomers. Nevertheless, saccharified waste potato starch can be used as a carbon source by *Ralstonia eutropha* NCIMB 11,599 to obtain high yields of PHB [31]. Production of PHB from starch by the recombinant *Corynebacterium glutamicum* strain was also demonstrated to yield 6.4% [65], and that from engineered *E. coli* SKB99 yielded 57.4% of cell dry weight [66].

The *Bacillus subtilis* strain was included in this study, due to the established production of extracellular α-amylase that efficiently hydrolyzes the starch [67]. This ability could actually be coupled to afford two-stage bioproducts such as biopolymers or biofuels accumulation [68]. Indeed, *B. subtilis* ATCC6633 achieved good growth on PVA/TPS and the highest growth on hydrolyzed PVA (Table 3, Table S1). In fact, all three strains could utilize hydrolyzed PVA as the sole carbon and energy source (Table S1, Figure S3). *B. subtilis* ATCC6633 and *Streptomyces* sp. JS520 were very efficient (substrate-to-biomass conversion of 53% and 76%, respectively) and JS520 could also produce undecylprodigiosin pigment from this carbon source (Figure S3).

3.2.2. PVA/TPS Degradation in Model Compost

The biodegradability of PVA/TPS films was tested in a model compost system at 37 °C. After 6 days, no change was observed except shrinking and the loss of elasticity. After 10 days, a change in color was observed along with visible cracks in the nontreated and bioaugmented compost samples, as well as orange circles in the heat-pretreated compost samples. After 20 days, discoloration was even more apparent in all samples (Figure 5). A weight loss of more than 30% was detected in all samples within 10 days, while samples in the heat-pretreated compost lost a further 13% wt over the subsequent 20 days (Table 4). Initially, the most efficient degradation occurred in the bioaugmented compost, suggesting that the addition of bacterial strains with a proven ability to hydrolyze and utilize PVA/TPS can be beneficial for the degradation process. However, upon prolonged incubation, the degradation in bioaugmented and heat-pretreated compost was comparable (Table 4). This may be due to the fact that the initial heat pretreatment of the compost favored sporulating microorganisms such as *Actinomyces* and fungi that required some time to develop.

Figure 5. PVA/TPA film samples after compost burial at different time points.

Table 4. Weight of washed and dried pieces of plastic bag after compost degradation. The weight of pieces prior to burial was approximately 38 mg.

Compost	Day 3	Day 10	Day 30	Weight Loss, %
Nontreated	31 ± 2 mg	22 ± 1 mg	22 ± 1 mg	42
Heat-pretreated	29 ± 1 mg	25 ± 2 mg	20 ± 1 mg	47
Bioaugmented	23 ± 1 mg	22 ± 1 mg	19 ± 1 mg	50

Previously, starch films degraded completely within 30–84 days, while cellulose, as a reference material, requires 10 days under the same conditions [24,25]. Notably, these investigations carried out composting at an elevated temperature of approximately 60 °C. More recently, the degradation of PVA/starch blend films by two strains isolated from compost has been reported. Strains *Bacillus* sp. DG22 and *Paenibacillus* sp. DG14 were found to degrade films to a comparable extent observed in the current study (45–75% depending on the type of blend used) [21]. A study investigating the degradation of a PVA/starch/glycerol blend with 20% PVA during composting revealed that the degradation of films was mostly due to starch and glycerol degradation, and that after 45 days of incubation, the PVA content was almost intact [69]. *B. subtilis* and *Aspergillus niger* were selected as amylase-producing microorganisms and used to assess the degradation of chemically modified starch/PVA blend films with different additives. The degradation rates were up to 60% for unmodified starch/PVA films and ranged from 50% to 25% for films with chemically modified starch, glycerol, and citric acid. A different study reported biodegradability (~90%) for 50% starch in PVA samples over 28 days [70].

FTIR analysis was employed to detect structural changes in the films over the degradation under different composting conditions (Figure 6). From the FTIR spectrum of the control PVA/TPS film, the presence of a broad peak in the area of 3200–3500 cm^{-1} can be attributed to the vibrational stretching of hydroxyl (–OH) groups, while the peak at wavenumbers between 2780 cm^{-1} and 2980 cm^{-1} was associated with C-H stretching. A sharp peak at 1711 cm^{-1} derived from the carbonyl -C=O functional group, and the low-intensity peak at 1650 cm^{-1} was due to the bound water [71]. The –CH$_2$ group characteristic vibrations appeared at wavenumbers of 1420 cm^{-1} and 840 cm^{-1}, while the absorption peak at 1300 cm^{-1} derived from the –C–O–C group deformation vibrations. Finally, the peak appearing in the area from 1090 to 1021 cm^{-1} was due to the stretching of aliphatic alcohols [72]. All the peaks visible in the FTIR spectrum of control PVA/TPS films came from both the PVA and starch, as the characteristic peaks of PVA and starch are similar [73,74]; therefore, they overlapped and could not be precisely distinguished. Furthermore, the broad absorption band in the area of 3200–3500 cm^{-1} was due to the inter- and intra-molecular hydrogen bonding of –OH groups in PVA and starch.
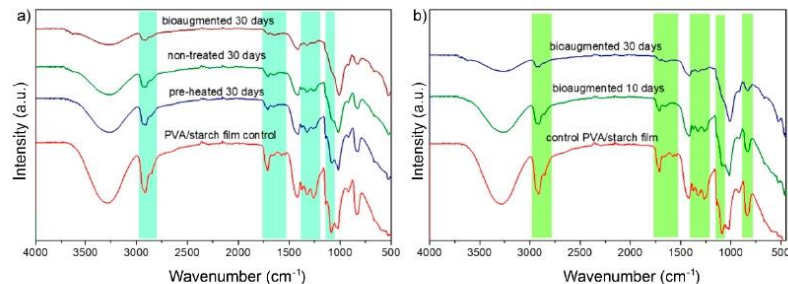

Figure 6. FTIR analysis of (**a**) degraded films after 30 days in different model composting systems, and (**b**) degraded films in bioaugmented composting system over time.

After degradation under different composting conditions, the structure of the degraded films was altered according to the FTIR spectra as the intensity of characteristic peaks decreased or completely dissipated, confirming the progressive degradation. The carbonyl peak intensity (at wavenumber of 1711 cm^{-1}) after 30 days of degradation significantly decreased in the case of all tested samples (Figure 6a), while in the case of bioaugmented testing conditions, this characteristic peak was hardly visible. A significant decrease in the peak intensity was also detected for the –CH$_2$ characteristic peak in the area from 2780 to 2980 cm^{-1}. The intensity of the broad absorption band coming from –OH groups decreased, but this peak also broadened, indicating that over the degradation course, the existing inter- and intra-molecular interactions between the –OH group of PVA and starch were disturbed. Further, the intensity of the –C–O–C characteristic band at the wavenumber of 1300 cm^{-1} decreased after degradation under nontreated and autoclaved conditions, while in the case of bioaugmented testing conditions, this peak disappeared. Decreasing peak intensity was also confirmed for the peaks in the area from 1090 to 1021 cm^{-1} (stretching vibrations in aliphatic alcohols) and at the wavenumber of 840 cm^{-1} (–CH$_2$ group vibrations). Comparing the degraded films under the different conditions, it could be assumed that films tested under bioaugmented and nontreated conditions indicated greater changes in structure in comparison to the PVA/TPS film tested under preheated conditions. Following the structural changes in the films observed over time from the representative FTIR spectrum (Figure 6b), it could be concluded that changes in the structure were more prominent over time. This was confirmed by the increase in weight loss, decrease in peak intensity, as well as dissipation of the characteristic peak after 30 days of degradation. All the characteristic peaks that were changed over time, decreased in peak intensity, or dissipated, under different testing conditions, changed in

the same manner for the films degraded under bioaugmented conditions, whereas the most prominent changes in the vibrations were observed at 2780-2980 cm^{-1} (–CH$_2$ vibrations), 1090 cm^{-1}, and 840 cm^{-1} (–CH$_2$ band vibrations).

The AFM micrographs of the control film and films composted under different conditions over time are presented in Figure 7. The polymer chains of PVA and starch in control samples formed basal planes due to the hydrogen bonds between those two hydrophilic polymers, and therefore, a planar surface with no irregularities was observed (light yellow color).
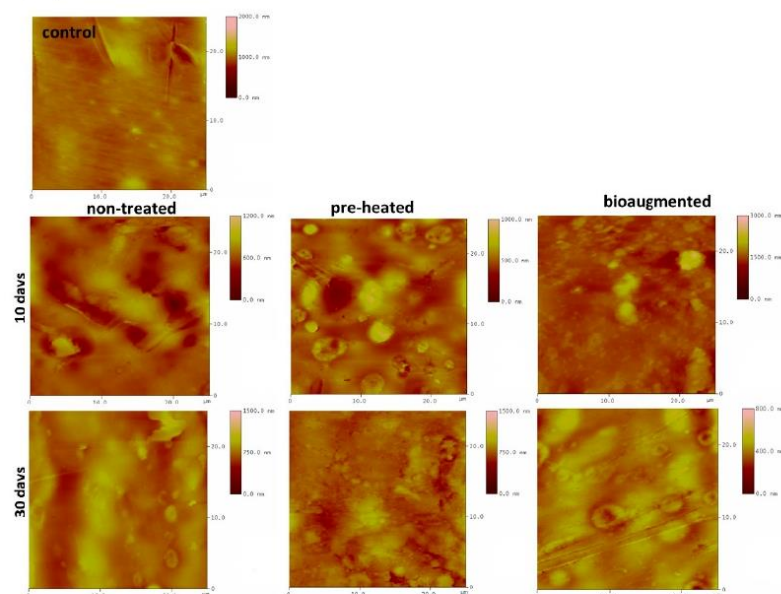
Figure 7. 2D AFM images, (25 × 25) μm, of PVA/TPS films before degradation (control) and after 3, 10, and 30 days of degradation under different model composting conditions.

The surface morphology was significantly altered after degradation in all three tested media, which was indicated by darker areas, representing higher elevations from the base of degraded films. Visual changes observed from 2D AFM images were supported by changes in surface roughness (RMS) measurements (Table 5). In the starting 10 days of degradation, RMS values increased in all three tested media with respect to the control sample (74.6 m), confirming surface erosion caused by enzymatic attack. After 30 days of composting, the RMS values were lower than those calculated for the 10 days of composting, but higher than those for the control sample for composting under preheated and nontreated conditions. This phenomenon could be explained by the starting erosion of the films that resulted in the increase in RMS values followed by the greater weight loss after 30 days of degradation when the films' surface was smoother as the thin layer of the films was eroded and the degradation products mostly leaked out of the remaining polymer matrix.

In order to gain an insight into the changes in morphology caused by the activity of enzymes presented in different composting systems, SEM analysis was also carried out and the recorded images of the PVA/TPS films before and after degradation are shown in Figure 8.

Table 5. AFM analysis results: RMS values of degraded films.

Sample *	RMS, nm 3 Days	RMS, nm 10 Days	RMS, nm 30 Days
PVA/starch film nontreated	104.7	100.3	98.8
PVA/starch film heat-pretreated	195.5	91.5	77.4
PVA/starch film bioaugmented	130.8	150.7	66.5

* RMS value of control sample was 74.6 nm.
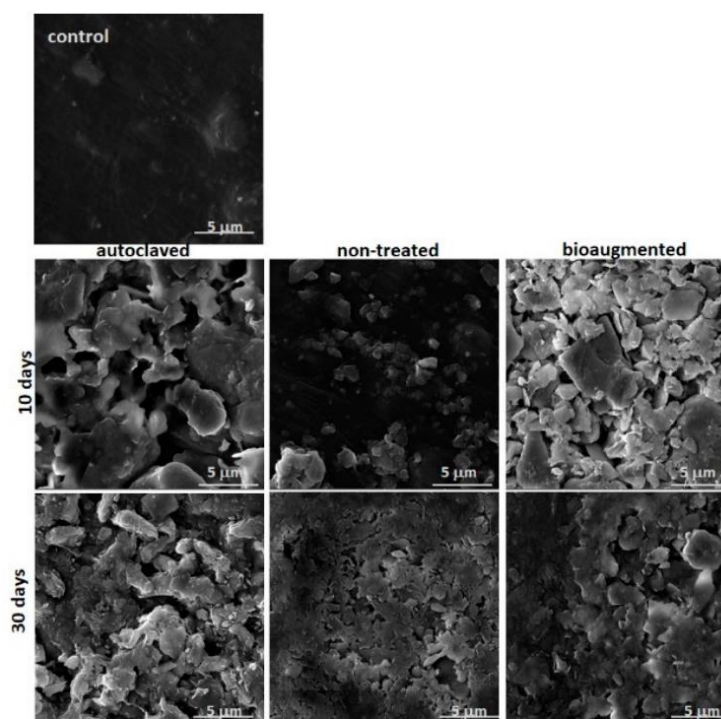
Figure 8. SEM images of PVA/TPS films after 10 and 30 days of composting under different conditions (magnification of 10,000×).

Pure PVA films appeared to have a planar, smooth surface and seemed to only possess visible agglomerates and granules after blending with starch [55]. Images of the control PVA/TPS film in Figure 8 indicated quite a smooth and planar surface morphology, with no visible signs of starch particles. After a degradation period of both 10 and 30 days, remarkable changes in surface morphology were detected. While those changes were less prominent in the case of films composted under nontreated conditions after 10 days, PVA/TPS films tested under preheated and bioaugmented conditions were highly disintegrated, and a lot of cracks and holes appeared on the surface. Observation of SEM images for the degraded samples clearly showed disintegration across entire films on the surface, indicated by a plethora of small crumbled/fractured parts, although the material strips remained somewhat consolidated overall but with significant weight loss. These results all point to the surface degradation mechanism so common for the enzymatic degradation that is, due to the activity of enzymes, excreted by the microbes present in the compost.

One of the desired properties of packaging materials is light barrier properties, especially for UV radiation. Light barrier properties of the PVA/TPS films before and after

composting under different conditions were observed in terms of the % transmittance (% *T*) for wavelengths in the range from 200 nm to 800 nm. As the % transmittance of investigated films in the UV region (200–400 nm) changed during degradation while the % transmittance remained constant in the visible region, changes in the % transmittance in the 200–400 nm UV region after 10 and 30 days of composting were calculated and are presented in Figure 9. The obtained results were in agreement with the captured photos of investigated films shown in Figure 5. In order to minimize the effect of compost material that might remain on the films due to degradation, which would further affect % transmittance measurements, the degraded films were washed with cold water. After 10 days of composting, the % *T* of preheated and bioaugmented samples decreased from 80% to approximately 45%. Over the same period, the decrease in % *T* for the nontreated sample was smaller, from 80% to 65%. The % *T* of the preheated and bioaugmented samples after 30 days was significantly reduced in comparison to the control sample, and decreases in % *T* of 44% and 27%, respectively, were detected. Changes in the % *T* were in line with the visible appearance of composted films (Figure 5), as after the degradation, PVA/TPS films became less transparent (Figure 9).
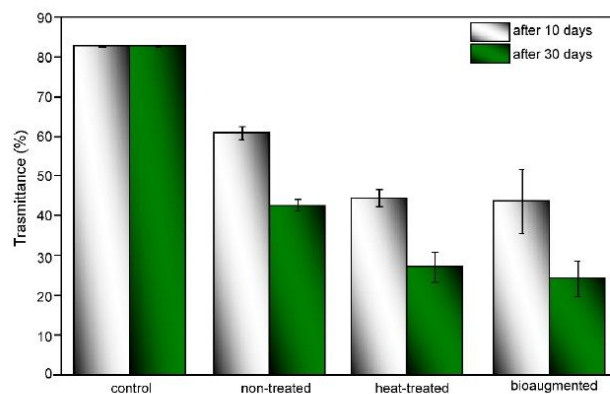
Figure 9. Changes in the % transmittance of the composted PVA/TPS films over time in the visible region from 200 to 400 nm.

4. Conclusions

In this study, low-energy, low-carbon post-use upcycling was demonstrated and presented as a model approach for PVA/TPS material circularity. The PVA/TPS film mechanical properties were comparable with those of LDPE and, hence, represent one of the improved mechanical performance and moisture sensitivity blends that have been developed recently [75]. Importantly, this work pioneers the conversion of the PVA/TPS film substrate to a high-market-value biopigment (undecylprodigiosin) at 5.3 mg/mL and/or PHB biopolyester at 7.8% of cell dry weight. The high degree of hydrophilicity and its solubility in water render this material an accessible carbon source for bioprocessing. Film amenability to biodegradation within compost systems was also demonstrated, with more than 50% weight loss occurring after 10 days at 37 °C. The upcycling approach described in this study has the potential to be instrumental in mitigating the damaging socioeconomic and environmental impacts of plastics production, increasing unabated, expected to double over the next 20 years.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/polym13213692/s1, Figure S1: Experimental setup of biodegradation under composting conditions: (a) model compost in Petri dishes, (b) PVA/starch film in model compost; Figure S2: UV-Vis spectrum of the extracted undecylprodigiosin from *Streptomyces* sp. JS520 grown in MSM medium

containing 10 g/L of PVA/TPS substrate and the appearance of the EtOAc culture extract; Figure S3: Pellets of centrifuged cultures grown in MSM medium with (A) 5 g/L of PVA and (B) 10 g/L of PVA. From left to right: *Ralstonia eutropha* H16, *Streptomyces* sp. JS520, and *B. subtilis* ATCC 6633; Table S1: Biomass yield (mg of dry mass/mL of culture) and conversion rate from bacterial cultures grown in MSM medium with PVA as sole carbon source.

References

1. Haider, T.P.; Völker, C.; Kramm, J.; Landfester, K.; Wurm, F.R. Plastics of the future? The impact of biodegradable polymers on the environment and on society. *Angew. Chem. Int. Ed.* **2019**, *58*, 50–62. [CrossRef]
2. Rameshkumar, S.; Shaiju, P.; O'connor, K.E.; P., R.B. Bio-based and biodegradable polymers—State-of-the-art, challenges and emerging trends. *Curr. Opin. Green Sus. Chem.* **2020**, *21*, 75–81. [CrossRef]
3. Blank, L.M.; Narancic, T.; Mampel, J.; Tiso, T.; O'connor, K. Biotechnological upcycling of plastic waste and other non-conventional feedstocks in a circular economy. *Curr. Opin. Biotechnol.* **2020**, *62*, 212–219. [CrossRef]
4. Nikolaivits, E.; Pantelic, B.; Azeem, M.; Taxeidis, G.; Babu, R.; Topakas, E.; Brennan Fournet, M.; Nikodinovic-Runic, J. Progressing plastics circularity: A review of mechano-biocatalytic approaches for waste plastic (re)valorization. *Front. Bioeng. Biotechnol.* **2021**, *9*, 535. [CrossRef]
5. Rommi, K.; Rahikainen, J.; Vartiainen, J.; Holopainen, U.; Lahtinen, P.; Honkapää, K.; Lantto, R. Potato peeling costreams as raw materials for biopolymer film preparation. *J. Appl. Poly. Sci.* **2016**, *133*, 42862. [CrossRef]
6. Fahrngruber, B.; Eichelter, J.; Erhäusl, S.; Seidl, B.; Wimmer, R.; Mundigler, N. Potato-fiber modified thermoplastic starch: Effects of fiber content on material properties and compound characteristics. *Eur. Polym. J.* **2019**, *111*, 170–177. [CrossRef]
7. Jane, J. Starch properties, modifications, and applications. *J. Macromol. Sci. Part A* **1995**, *32*, 751–757. [CrossRef]
8. Tian, H.; Yan, J.; Rajulu, A.V.; Xiang, A.; Luo, X. Fabrication and properties of polyvinyl alcohol/starch blend films: Effect of composition and humidity. *Int. J. Biol. Macromol.* **2017**, *96*, 518–523. [CrossRef] [PubMed]
9. Jeremic, S.; Milovanovic, J.; Mojicevic, M.; Bogojevic, S.S.; Nikodinovic-Runic, J. Understanding bioplastic materials–Current state and trends. *J. Serb. Chem. Soc.* **2020**, *85*, 1507–1538. [CrossRef]
10. Afzal, A.; Khaliq, Z.; Ahmad, S.; Ahmad, F.; Noor, A.; Qadir, M.B. Development and characterization of biodegradable composite film. *Environ. Technol. Innov.* **2021**, *23*, 101664. [CrossRef]
11. Mohd Asharuddin, S.; Othman, N.; Altowayti, W.A.H.; Abu Bakar, N.; Hassan, A. Recent advancement in starch modification and its application as water treatment agent. *Environ. Technol. Innov.* **2021**, *23*, 101637. [CrossRef]
12. Chiellini, E.; Corti, A.; D'antone, S.; Solaro, R. Biodegradation of poly (vinyl alcohol) based materials. *Prog. Polym. Sci.* **2003**, *28*, 963–1014. [CrossRef]
13. Ge, C.; Lansing, B.; Lewis, C.L. Thermoplastic starch and poly(vinyl alcohol) blends centered barrier film for food packaging applications. *Food Packag. Shelf Life* **2021**, *27*, 100610. [CrossRef]
14. Srivastava, K.R.; Dixit, S.; Pal, D.B.; Mishra, P.K.; Srivastava, P.; Srivastava, N.; Hashem, A.; Alqarawi, A.A.; Abd_Allah, E.F. Effect of nanocellulose on mechanical and barrier properties of PVA–banana pseudostem fiber composite films. *Environ. Technol. Innov.* **2021**, *21*, 101312. [CrossRef]
15. Gadhave, R.V.; Das, A.; Mahanwar, P.A.; Gadekar, P.T. Starch based bio-plastics: The future of sustainable packaging. *Open Polymer Chem.* **2018**, *8*, 21–33. [CrossRef]
16. Bangar, S.P.; Purewal, S.S.; Trif, M.; Maqsood, S.; Kumar, M.; Manjunatha, V.; Rusu, A.V. Functionality and applicability of starch-based films: An eco-friendly approach. *Foods* **2021**, *10*, 2181. [CrossRef]
17. Manikandan, N.A.; Pakshirajan, K.; Pugazhenthi, G. Techno-economic assessment of a sustainable and cost-effective bioprocess for large scale production of polyhydroxybutyrate. *Chemosphere* **2021**, *284*, 131371. [CrossRef] [PubMed]

18. Sakai, K.; Hamada, N.; Watanabe, Y. Studies on the poly (vinyl alcohol) degrading enzyme. Part VI. Degradation mechanism of poly (vinyl alcohol) by successive reactions of secondary alcohol oxidase and. BETA.-diketone hydrolase from *Pseudomonas* sp. *Agric. Biol. Chem.* **1986**, *50*, 989–996. [CrossRef]

19. Suzuki, T. Degradation of poly (vinyl alcohol) by microorganisms. *Appl. Polym. Symp.* **1979**, *35*, 431–437.

20. Shimao, M.; Fujita, I.; Kato, N.; Sakazawa, C. Enhancement of pyrroloquinoline quinone production and polyvinyl alcohol degradation in mixed continuous cultures of Pseudomonas putida VM15A and *Pseudomonas* sp. strain VM15C with mixed carbon sources. *Appl. Environ. Microbiol.* **1985**, *49*, 1389. [CrossRef]

21. Liu, Y.; Deng, Y.; Chen, P.; Duan, M.; Lin, X.; Zhang, Y. Biodegradation analysis of polyvinyl alcohol during the compost burial course. *J. Basic Microbiol.* **2019**, *59*, 368–374. [CrossRef]

22. Mori, T.; Sakimoto, M.; Kagi, T.; Sakai, T. Isolation and characterization of a strain of *Bacillus megaterium* that degrades poly (vinyl alcohol). *Biosci. Biotechnol. Biochem.* **1996**, *60*, 330–332. [CrossRef]

23. Kliem, S.; Kreutzbruck, M.; Bonten, C. Review on the biological degradation of polymers in various environments. *Materials* **2020**, *13*, 4586. [CrossRef] [PubMed]

24. Vikman, M.; Itävaara, M.; Poutanen, K. Biodegradation of starch-based materials. *J. Macromol. Sci. A* **1995**, *32*, 863–866. [CrossRef]

25. Torres, F.G.; Troncoso, O.P.; Torres, C.; Díaz, D.A.; Amaya, E. Biodegradability and mechanical properties of starch films from Andean crops. *Int. J. Biol. Macromol.* **2011**, *48*, 603–606. [CrossRef] [PubMed]

26. Yun, Y.-H.; Wee, Y.-J.; Byun, H.-S.; Yoon, S.-D. Biodegradability of chemically modified starch (RS4)/PVA blend films: Part 2. *J. Polym. Environ.* **2008**, *16*, 12–18. [CrossRef]

27. Kalia, V.C.; Patel, S.K.S.; Shanmugam, R.; Lee, J.-K. Polyhydroxyalkanoates: Trends and advances toward biotechnological applications. *Biores. Technol.* **2021**, *326*, 124737. [CrossRef]

28. Mcadam, B.; Brennan Fournet, M.; Mcdonald, P.; Mojicevic, M. Production of polyhydroxybutyrate (PHB) and factors impacting its chemical and mechanical characteristics. *Polymers* **2020**, *12*, 2908. [CrossRef]

29. Tang, R.; Weng, C.; Peng, X.; Han, Y. Metabolic engineering of *Cupriavidus necator* H16 for improved chemoautotrophic growth and PHB production under oxygen-limiting conditions. *Metab. Eng.* **2020**, *61*, 11–23. [CrossRef]

30. Kim, B.S. Production of poly (3-hydroxybutyrate) from inexpensive substrates. *Enzyme Microb. Tech.* **2000**, *27*, 774–777. [CrossRef]

31. Haas, R.; Jin, B.; Zepf, F.T. Production of poly (3-hydroxybutyrate) from waste potato starch. *Biosci. Biotechnol. Biochem.* **2008**, *72*, 253–256. [CrossRef] [PubMed]

32. Venil, C.K.; Dufossé, L.; Renuka Devi, P. Bacterial Pigments: Sustainable Compounds with Market Potential for Pharma and Food Industry. *Front. Sus. Food Syst.* **2020**, *4*, 100. [CrossRef]

33. Stankovic, N.; Senerovic, L.; Ilic-Tomic, T.; Vasiljevic, B.; Nikodinovic-Runic, J. Properties and applications of undecylprodigiosin and other bacterial prodigiosins. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 3841–3858. [CrossRef] [PubMed]

34. Mittal, A.; Garg, S.; Bajpai, S. Thermal decomposition kinetics and properties of grafted barley husk reinforced PVA/starch composite films for packaging applications. *Carbohyd. Polym.* **2020**, *240*, 116225. [CrossRef]

35. Costa, N.N.; De Faria Lopes, L.; Ferreira, D.F.; De Prado, E.M.L.; Severi, J.A.; Resende, J.A.; De Paula Careta, F.; Ferreira, M.C.P.; Carreira, L.G.; De Souza, S.O.L. Polymeric films containing pomegranate peel extract based on PVA/starch/PAA blends for use as wound dressing: In vitro analysis and physicochemical evaluation. *Mat. Sci. Eng. C* **2020**, *109*, 110643. [CrossRef]

36. Flores, S.K.; Costa, D.; Yamashita, F.; Gerschenson, L.N.; Grossmann, M.V. Mixture design for evaluation of potassium sorbate and xanthan gum effect on properties of tapioca starch films obtained by extrusion. *Mat. Sci. Eng. C* **2010**, *30*, 196–202. [CrossRef]

37. Anglès, M.N.; Dufresne, A. Plasticized starch/tunicin whiskers nanocomposites. 1. Structural analysis. *Macromolecules* **2000**, *33*, 8344–8353. [CrossRef]

38. Stankovic, N.; Radulovic, V.; Petkovic, M.; Vuckovic, I.; Jadranin, M.; Vasiljevic, B.; Nikodinovic-Runic, J. *Streptomyces* sp. JS520 produces exceptionally high quantities of undecylprodigiosin with antibacterial, antioxidative, and UV-protective properties. *Appl. Microbiol. Biotechnol.* **2012**, *96*, 1217–1231. [CrossRef] [PubMed]

39. Pohlmann, A.; Fricke, W.F.; Reinecke, F.; Kusian, B.; Liesegang, H.; Cramm, R.; Eitinger, T.; Ewering, C.; Pötter, M.; Schwartz, E.; et al. Genome sequence of the bioplastic-producing "Knallgas" bacterium *Ralstonia eutropha* H16. *Nature Biotechnol.* **2006**, *24*, 1257–1262. [CrossRef]

40. Schlegel, H.G.; Kaltwasser, H.; Gottschalk, G. A submersion method for culture of hydrogen-oxidizing bacteria: Growth physiological studies. *Arch. Mikrobiol.* **1961**, *38*, 209–222. [CrossRef]

41. Juengert, J.R.; Bresan, S.; Jendrossek, D. Determination of polyhydroxybutyrate (PHB) content in *Ralstonia eutropha* using gas chromatography and Nile Red staining. *Bio-Protocol* **2018**, *8*, e2748. [CrossRef] [PubMed]

42. Ponjavic, M.; Nikolic, M.S.; Nikodinovic-Runic, J.; Jeremic, S.; Stevanovic, S.; Djonlagic, J. Degradation behaviour of PCL/PEO/PCL and PCL/PEO block copolymers under controlled hydrolytic, enzymatic and composting conditions. *Polym. Test.* **2017**, *57*, 67–77. [CrossRef]

43. Jose, J.; Al-Harthi, M.A.; Alma'adeed, M.a.A.; Bhadra Dakua, J.; De, S.K. Effect of graphene loading on thermomechanical properties of poly (vinyl alcohol)/starch blend. *J. Appl. Poly. Sci.* **2015**, *132*, 41827. [CrossRef]

44. Liu, X.; Yu, L.; Liu, H.; Chen, L.; Li, L. In situ thermal decomposition of starch with constant moisture in a sealed system. *Poly. Deg. Stabil.* **2008**, *93*, 260–262. [CrossRef]

45. Guerrini, L.M.; De Oliveira, M.P.; Branciforti, M.C.; Custódio, T.A.; Bretas, R.E. Thermal and structural characterization of nanofibers of poly (vinyl alcohol) produced by electrospinning. *J. Appl. Poly. Sci.* **2009**, *112*, 1680–1687. [CrossRef]

46. Cassu, S.N.; Felisberti, M.I. Poly (vinyl alcohol) and poly (vinyl pyrrolidone) blends: Miscibility, microheterogeneity and free volume change. *Polymer* **1997**, *38*, 3907–3911. [CrossRef]

47. Zhang, M.; Cheng, Z.; Zhao, T.; Liu, M.; Hu, M.; Li, J. Synthesis, characterization, and swelling behaviors of salt-sensitive maize bran–poly (acrylic acid) superabsorbent hydrogel. *J. Agri. Food Chem.* **2014**, *62*, 8867–8874. [CrossRef]

48. Jain, N.; Singh, V.K.; Chauhan, S. A review on mechanical and water absorption properties of polyvinyl alcohol based composites/films. *J. Mech. Behav. Mat.* **2017**, *26*, 213–222. [CrossRef]

49. Musa, B.H.; Hameed, N.J. Study of the mechanical properties of polyvinyl alcohol/starch blends. *Mat. Today Proceed.* **2020**, *20*, 439–442. [CrossRef]

50. Guohua, Z.; Ya, L.; Cuilan, F.; Min, Z.; Caiqiong, Z.; Zongdao, C. Water resistance, mechanical properties and biodegradability of methylated-cornstarch/poly (vinyl alcohol) blend film. *Poly. Deg. Stabil.* **2006**, *91*, 703–711. [CrossRef]

51. Massey, L.K. *Film Properties of Plastics and Elastomers*; Elsevier: Amsterdam, The Netherlands, 2004.

52. Yan, C.; Li, H.; Zhang, X.; Zhu, Y.; Fan, X.; Yu, L. Preparation and properties of continuous glass fiber reinforced anionic polyamide-6 thermoplastic composites. *Mater. Design* **2013**, *46*, 688–695. [CrossRef]

53. Domene-López, D.; García-Quesada, J.C.; Martin-Gullon, I.; Montalbán, M.G. Influence of starch composition and molecular weight on physicochemical properties of biodegradable films. *Polymers* **2019**, *11*, 1084. [CrossRef] [PubMed]

54. Abdullah, Z.W.; Dong, Y. Biodegradable and water resistant poly(vinyl alcohol) (PVA)/starch (ST)/glycerol (GL)/halloysite nanotube (HNT) nanocomposite films for sustainable food packaging. *Front. Mater.* **2019**, *6*, 58. [CrossRef]

55. Mittal, A.; Garg, S.; Bajpai, S. Fabrication and characteristics of poly (vinyl alcohol)-starch-cellulosic material based biodegradable composite film for packaging application. *Mat. Today Proc.* **2020**, *21*, 1577–1582. [CrossRef]

56. Nikodinovic-Runic, J.; Guzik, M.; Kenny, S.T.; Babu, R.; Werker, A.; Ke, O.C. Carbon-rich wastes as feedstocks for biodegradable polymer (polyhydroxyalkanoate) production using bacteria. *Adv. Appl. Microbiol.* **2013**, *84*, 139–200. [PubMed]

57. Sohn, Y.J.; Kim, H.T.; Baritugo, K.A.; Jo, S.Y.; Song, H.M.; Park, S.Y.; Park, S.K.; Pyo, J.; Cha, H.G.; Kim, H. Recent advances in sustainable plastic upcycling and biopolymers. *Biotechnol. J.* **2020**, *15*, 1900489. [CrossRef]

58. Tiso, T.; Narancic, T.; Wei, R.; Pollet, E.; Beagan, N.; Schröder, K.; Honak, A.; Jiang, M.; Kenny, S.T.; Wierckx, N. Towards bio-upcycling of polyethylene terephthalate. *Metab. Eng.* **2021**, *66*, 167–178. [CrossRef]

59. Yip, C.H.; Yarkoni, O.; Ajioka, J.; Wan, K.L.; Nathan, S. Recent advancements in high-level synthesis of the promising clinical drug, prodigiosin. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 1667–1680. [CrossRef]

60. Merck. Undecylprodigiosin hydrochloride. Available online: https://www.sigmaaldrich.com/rs/en/product/sigma/sml1576 (accessed on 23 August 2021).

61. Luti, K.J.K. Mixture design of experiments for the optimization of carbon source for promoting undecylprodigiosin and actinorhodin production. *J. Pure Appl. Microbiol.* **2018**, *12*, 1783–1794. [CrossRef]

62. Luti, K.; Younis, R. An induction of undecylprodigiosin production from *Streptomyces coelicolor* by elicitation with microbial cells using solid state fermentation. *Iraqi J. Sci.* **2014**, *55*, 553–1562.

63. Nguyen, T.-H.; Wang, S.-L.; Nguyen, D.-N.; Nguyen, A.-D.; Nguyen, T.-H.; Doan, M.-D.; Ngo, V.-A.; Doan, C.-T.; Kuo, Y.-H.; Nguyen, V.-B. Bioprocessing of marine chitinous wastes for the production of bioactive prodigiosin. *Molecules* **2021**, *26*, 3138. [CrossRef]

64. Volodina, E.; Raberg, M.; Steinbüchel, A. Engineering the heterotrophic carbon sources utilization range of Ralstonia eutropha H16 for applications in biotechnology. *Crit. Rev. Biotechnol.* **2016**, *36*, 978–991. [CrossRef]

65. Song, Y.; Matsumoto, K.I.; Tanaka, T.; Kondo, A.; Taguchi, S. Single-step production of polyhydroxybutyrate from starch by using α-amylase cell-surface displaying system of *Corynebacterium glutamicum*. *J. Biosci. Bioeng.* **2013**, *115*, 12–14. [CrossRef]

66. Bhatia, S.K.; Shim, Y.-H.; Jeon, J.-M.; Brigham, C.J.; Kim, Y.-H.; Kim, H.-J.; Seo, H.-M.; Lee, J.-H.; Kim, J.-H.; Yi, D.-H.; et al. Starch based polyhydroxybutyrate production in engineered *Escherichia coli*. *Bioprocess Biosyst. Eng.* **2015**, *38*, 1479–1484. [CrossRef]

67. Konsula, Z.; Liakopoulou-Kyriakides, M. Hydrolysis of starches by the action of an α-amylase from *Bacillus subtilis*. *Process Biochem.* **2004**, *39*, 1745–1749. [CrossRef]

68. Tran, H.T.M.; Cheirsilp, B.; Hodgson, B.; Umsakul, K. Potential use of Bacillus subtilis in a co-culture with *Clostridium butylicum* for acetone–butanol–ethanol production from cassava starch. *Biochem. Eng. J.* **2010**, *48*, 260–267. [CrossRef]

69. Jayasekara, R.; Harding, I.; Bowater, I.; Christie, G.B.; Lonergan, G.T. Biodegradation by composting of surface modified starch and PVA blended films. *J. Polym. Environ.* **2003**, *11*, 49–56. [CrossRef]

70. Thakur, K.; Rajhans, A.; Kandasubramanian, B. Starch/PVA hydrogels for oil/water separation. *Environ. Sci. Pollut. Res.* **2019**, *26*, 32013–32028. [CrossRef] [PubMed]

71. Wolkers, W.F.; Oliver, A.E.; Tablin, F.; Crowe, J.H. A Fourier-transform infrared spectroscopy study of sugar glasses. *Carbohyd. Res.* **2004**, *339*, 1077–1085. [CrossRef] [PubMed]

72. Pirzada, T.; Arvidson, S.A.; Saquing, C.D.; Shah, S.S.; Khan, S.A. Hybrid silica–PVA nanofibers via sol–gel electrospinning. *Langmuir* **2012**, *28*, 5834–5844. [CrossRef]

73. Lima, E.; Raphael, E.; Sentanin, F.; Rodrigues, L.; Ferreira, R.; Carlos, L.; Silva, M.; Pawlicka, A. Photoluminescent polymer electrolyte based on agar and containing europium picrate for electrochemical devices. *Mat. Sci. Eng. B* **2012**, *177*, 488–493. [CrossRef]

74. Guan, Y.; Qian, L.; Xiao, H.; Zheng, A. Preparation of novel antimicrobial-modified starch and its adsorption on cellulose fibers: Part I. Optimization of synthetic conditions and antimicrobial activities. *Cellulose* **2008**, *15*, 609–618. [CrossRef]

75. Jiang, T.; Duan, Q.; Zhu, J.; Liu, H.; Yu, L. Starch-based biodegradable materials: Challenges and opportunities. *Adv. Ind. Eng. Polym. Res.* **2020**, *3*, 8–18. [CrossRef]

4. DISKUSIJA

4.1. Izolovani mikroorganizmi kao potencijalni biokatalizatori za biodegradaciju plastike

Zajedno sa plastičnim otpadom skoro svaki ekosistem sadrži i mikroorganizme sa sposobnošću razgradnje plastike (Herrera et al., 2023). U potrazi za ovim mikroorganizmima razvijeni su jednostavni testovi na Petrijevim šoljama koji koriste podloge za gajenje mikroorganizama sa plastičnim polimerima, njihovim oligomerima i monomerima kao jedinim izvorom ugljenika (Molitor et al., 2020). Ovakvi testovi omogućili su jednostavnu selekciju i izolaciju mikroorganizama koji potencijalno mogu biti upotrebljeni za razgradnju plastike (Charnock, 2021, Rüthi et al., 2023, Ciric et al., 2024). Osnovni cilj ove doktorske disertacije bio je izolacija i karakterizacija bakterijskih sojeva i njihovih enzima koji se mogu iskoristiti u razgradnji i valorizaciji plastičnog otpada. Kako bi se to postiglo, kao prvi korak ovakvi testovi visoke propusnosti korišćeni su za određivanje potencijala 220 mikroorganizama iz različitih staništa za razgradnju plastike (odeljak 3.2. str. 39). Uzimajući u obzir da su zemljište i deponije najbogatiji izvori mikroorganizama koji razgrađuju plastiku (Gambarini et al., 2021), mikroorganizmi izolovani u okviru ove disertacije izolovani su iz zagađenog i nezagađenog zemljišta. Zemljište zagađeno plastičnim otpadom korišćeno je u cilju povećavanja verovatnoće izolovanja mikroorganizama iz „plastisfere" koja predstavlja zajednicu mikroorganizama na i u blizini plastike različitog sastava od okolnog mikrobioma (Park et al., 2023). Na osnovu rezultata dobijenih u okviru ove doktorske disertacije najveći broj mikroorganizama (~5%) pokazao je potencijal za razgradnju PU (odeljak 3.2. i 3.3) dok značajne razlike između mikroorganizama iz zagađenih i nezagađenih staništa nisu uočene. Dodatno, kako bi se ispitao uticaj PU otpada na diverzitet mikroorganizama sprovedena je metagenomska analiza sastava zajednice mikroorganizama *in vitro* (odeljak 3.3. str. 60). Ovom analizom detektovano je samo blago smanjenje diverziteta u zemljištu zagađenom plastikom nakon 18 meseci inkubacije. Smanjenje diverziteta kao odgovor na zagađenje plastikom uočeno je i u slučaju PE, PLA i poli(butilen-adipat-*co*-tereftalat), a stepen promene zavisio je od tipa plastike i njene podložnosti razgradnji (Rüthi et al., 2023). Međutim, s obzirom da su slične promene uočene i u kontrolnim uzorcima (zemljište inkubirano bez prisustva PU materijala) razlike u diverzitetu uzrokovane PU otpadom najverovatnije su manje izražene od promena izazvanih inkubacionim uslovima. Ukazujući na činjenicu da uslovi kao što su temperatura, vlažnost i pH vrednost znatno više utiču na diverzitet mikroorganizama od plastičnog otpada. Ovo je u skladu i sa novijim istraživanjima koja pronalaze visoko efikasne PETaze u ekstremnim staništima, a ne plastikom zagađenim staništima (Sonnendecker et al., 2022, Erickson et al., 2022)

Shodno rezultatima dobijenim iz sličnih istraživanja sprovedenih u Norveškoj, Irskoj i Švajcarskoj najveći broj mikroorganizama koji su pokazali potencijal za razgradnju plastike, iz uzoraka korišćenih u ovoj disertaciji, pripadao je rodovima *Bacillus* i *Streptomyces* bez značajnih razlika između zagađenih i nezagađenih staništa (Herrera et al., 2023, Charnock, 2021, Rüthi et al., 2023). Herrera i saradnici identifikovali su *Streptomyces*, *Bacillus*, *Enterococcus* i *Pseudomonas* sojeve kao najpotentnije za razgradnju hidrolizabilnih polimera (Herrera et al.,

2023). Generalno, sojevi rodova *Rhodococcus* i *Streptomyces* često su identifikovani kao zemljišni mikroorganizmi sposobni da razrađuju PET (Charnock, 2021). Dok su Ruthi i saradnici su, pored bakterija iz malo poznatog razdela Saccharibacteria, takođe utvrdili da su *Streptomyces*, *Amycolatopsis* i *Rhodococcus* sojevi dominantni taksoni plastisfere kao konvencionale tako i biorazgradive plastike u hladnim krajevima (Rüthi et al., 2023). Najaktivniji sojevi identifikovani u ovom istraživanju odabrani su za dalje eksperimente i detaljniju karakterizaciju mehanizama razgradnje različitih polimera.

4.2. Primena *B. subtilis* BPM12 u biodegradaciji poli(etilentereftalata)

Nakon pretraživanja laboratorijske kolekcije od 220 mikroorganizama, *B. subtilis* BPM12 izabran je kao kandidat za razgradnju PET s obzirom da je rastao i formirao zone prosvetljenja na čvrstim podlogama sa BHET kao jedinim izvorom ugljenika (odeljak 3.1. str. 23). Rod *Bacillus* je vrlo dobro izučen, sa brojnim predstavnicima identifikovanim kao biokatalizatorima sposobnim za razgradnju ksenobiotika (Gangola et al., 2018). Dodatno, *Bacillus* sojevi su takođe identifikovani u bakterijskim konzorcijumima koji razgrađuju različite vrste plastike (Roberts et al., 2020, Shah et al., 2016), a pokazano je i da pojedinačni sojevi imaju sposobnost razgradnje PET, PU i PLA (Ribitsch et al., 2011, Shah et al., 2013b, Bonifer et al., 2019). Visoka tolerancija na sredinske faktore kao što su temperatura i pH vrednost i ustaljeni protokoli za genetičku manipulaciju čine *Bacillus* vrlo privlačnim za biotehnološke aplikacije (Hanim, 2017, Wang et al., 2019). *B. subtilis* BPM12 takođe je pokazao vrlo poželjne karakteristike rastući na temperaturama preko 40°C i pH vrednostima od pH 5 do pH 10.

Koristeći ćelije *B. subtilis* BPM12 ispitana je preferencija ovog soja prema različitim gradivnim jedinicama PET (monomerima i oligomerima), sintetisanih u svrhu lakše karakterizacije biokatalizatora za razgradnju PET (Djapovic et al., 2021). Monomere su predstavljali metil- i etil-estri TPA, a oligomere su predstavljali dva do tri molekula TPA povezana etilen-glikolom (odeljak 3.1. str. 24). Utvrđeno je da *B. subtilis* BPM12 razgrađuje BHET efikasnije od istaknutih sojeva za razgradnju BHET *Enterobacter* sp. HY1 i *Y. lipolytica* IMUFRJ 50682 (Qiu et al., 2020, da Costa et al., 2020). Međutim, manje efikasno od rekombinantnih *Y. lipolytica* Po1f i *B. subtilis* 168 sojeva transformisanih sa IsPETazom (Liu et al., 2021b, Qi et al., 2021). HPLC analizom utvrđeno je da BHET prvenstveno biva hidrolizovan u MHET, a zatim, znatno sporije, MHET biva hidrolizovan do TPA. Ovakva dinamika, koja podrazumeva prvo hidrolizu BHET, a zatim znatno sporiju hidrolizu MHET uočena je kod različitih enzima uključenih u razgradnju PET. Prvenstveno se pripisuje inhibiciji enzima proizvodom, s obzirom da je pokazano da MHET inhibira aktivnost PETaza uključujući i PETaze poreklom iz *Bacillus* kao što je enzim Bs2Est (Kim et al., 2021, Mrigwani et al., 2022).

Dalja istraživanja otkrila su da *B. subtilis* BPM12 pokazuje i egzo- i endohidrolaznu aktivnost prema estarskim vezama sadržanim u monomerima i oligomerima PET. Kada je u pitanju egzohidrolazna aktivnost, prvenstveno je raskidana veza između TPA i etil-grupe, dok je veza između TPA i metil-grupe hidrolizovana u znatno manjoj meri. U oligomerima, veza između dve

jedinice TPA efikasno je raskidana endohidrolaznom aktivnošću. Sa druge strane niska rastvorljivost oligomera sa tri jedinice TPA praktično je onemogućila reakciju i samo tragovi degradacionih produkata su detektovani. Sličan mehanizam aktivnosti koji uključuje prvo hidrolizu estarskih veza između dva molekula TPA, a zatim hidrolizu do manjih molekula kao što su MHET i TPA pokazan je i kod Bs2Est (Kim et al., 2021). Dodatno, ovaj set PET monomera i oligomera korišćen je i za karakterizaciju PETaze iz *Moraxella* sp., koja se pokazala vrlo efikasnom u razgradnji PET visoke kristaliničnosti, a imala je isti profil razgradnje oligomera kao i *B. subtilis* BPM12 (Nikolaivits et al., 2022).

Sposobnost formiranja biofilma se prilikom upotrebe celih ćelija kao biokatalizatora za razgradnju plastike pokazala kao važan faktor koji utiče na efikasnost razgradnje (odeljak 1.2, **Slika 2**). Visok nivo hidrofobnosti plastike onemogućava efikasno prianjanje mikroorganizama i adsorpciju ezima i time smanjuje efikasnost razgradnje. Tako mikroorganizmi koji mogu da formiraju biofilm na plastici dovode u bliski kontakt enzimsku mašineriju odgovornu za razgradnju plastike i stvaraju mikro okruženje pogodno za razgradnju (Howard and McCarthy, 2023). Iz tog razloga ispitana je sposobnost formiranja biofilma *B. subtilis* BPM12 na PET filmovima (odeljak 3.1. str. 28). Uporedo, ispitana je i sposobnost formiranja biofilma ovog soja na mehanički recikliranim PET filmovima, s obzirom da je sve veći udeo recikliranog PET u otpadnim tokovima. *B. subtilis* BPM12 je uspešno formirao biofilm na svim testiranim PET filmovima. Reciklirani filmovi bili su primetno pogodniji za formiranje biofilma, najverovatnije zbog promena na površini filmova odnosno neravnina nastalih nakon mehaničkog recikliranja. Nažalost, formiranje biofilma nije bilo praćeno i smanjenjem mase i morfološkim promenama PET materijala pod testiranim uslovima. Iako je prianjanje mikroorganizama za plastiku jedan od preduslova za efikasnu razgradnju, formiranje biofilma na različitim vrstama plastike nije uvek povezana sa njenom razgradnjom. Dokazano je da vrste iz razdela Proteobacteria i Bacteroidetes predstavljaju dominantne mikroorganizme biofilmova formiranih na PE i najlonu ali da ne dovode do razgradnje ovih polimera (Wallbank et al., 2022).

Upotrebom proteinskog ekstrakta *B. subtilis* BPM12 došlo je do morfoloških promena površine PET filmova nalik onim uočenim u studiji sprovedenoj od strane Chen i saradnika (Chen et al., 2022). Ove promene impliciraju površinsku razgradnju PET filma koja je dodatno potvrđena detektovanjem TPA i MHET u rastvoru (odeljak 3.1. str. 28). Bitno je napomenuti da PET film koji je imao najniži stepen kristaliničnosti je ujedno i onaj koji je pokazao najjasnije promene. Dokazano je da kristaliničnost igra ključnu ulogu u efikasnoj razgradnji PET čak i kod najpotentnijih enzima. Niska kristaliničnost podrazumeva više amorfnih regiona za koje se smatra da su pogodniji za biološku razgradnju zato što omogućavaju bolji pristup enzima (Thomsen et al., 2022, Tournier et al., 2020).

4.2.1. Enzim Bpm12CE efikasno razgrađuje gradivne jedinice poli(etilentereftalata)

Uprkos niskoj efikasnosti razgradnje PET polimera, sposobnost *B. subtilis* BPM12 da hidrolizuje različite PET monomere i oligomere ukazuje na prisustvo enzima koji mogu biti korisni

u razgradnji PET. Kombinacije PETaza sa enzimima koji razgrađuju monomere PET pokazale su se kao korisna strategija. U ovakvim sistemima PETaza razgrađuje polimerne lance na monomere i oligomere dok dodatni enzimi deluju na monomere i oligomere smanjujući kompeticiju za vezivanje za aktivno mesto PETaze i time povećavaju efikasnost razgradnje (Barth et al., 2016). Upotrebom sistema koji se sastojao od LCC PETaze i karboksilesteraze iz *Thermus thermophilus* postignuto je i do 100% povećanje u prinosu TPA prilikom razgradnje PET (Mrigwani et al., 2022). Stoga je pristupljeno genomskoj analizi *B. subtilis* BPM12 kako bi se identifikovali enzimi koji su odgovorni za aktivnost ovog soja na PET monomerima i oligomerima kao i samom PET polimeru.

Proteom *B. subtilis* BPM12 upoređen je sa proteomima *Bacillus* sojeva koji dokazano razgrađuju PET i PAZy bazom enzima koji razgrađuju plastiku (odeljak 3.1. str. 25). *B. thuringiensis* C15 i *B. albus* PFZN01 su deo konzorcijuma koji se sastoji od pet sojeva sposobnih da rastu na PET kao jedinom izvoru ugljenika koristeći oslobođene monomere za rast (Roberts et al., 2020). *Bacillus* sp. AIIW2, slično *B. subtilis* BPM12, formirao je biofilm na PET filmovima i dovodio do površinskih promena materijala (Kumari et al., 2021). Skoro polovina proteina *B. subtilis* BPM12 imaju svoje homologe u proteomima tri soja prijavljena da razgrađuju PET, najviše sa *Bacillus* sp. AIIW2. Prema transkriptomskoj analizi soja AIIW2 gajenog u prisustvu PET, protein sa najintenzivnijom promenom ekspresije bila je unutarćelijska karboksilesteraza (WP_020451834.1) koja ima 68% identičnosti sa alfa/beta hidrolazom (WP_014480039.1) iz *B. subtilis* BPM12. Ova α/β hidrolaza identifikovana je kao enzim potencijalno odgovoran za hidrolizu PET oligomera od strane *B. subtilis* BPM12. Međutim, kao najbolji kandidat pokazala se karboksilesteraza nazvana Bpm12CE koja ima 98% identičnosti sa PETazom iz *B. subtilis* 4P3-11 koja hidrolizuje PET filmove, ali ujedno može i da hidrolizuje PET trimere (Ribitsch et al., 2011). Ova pretpostavka je potvrđena delecijom *bpm12CE* gena čime je *B. subtilis* BPM12 izgubio sposobnost da raste na BHET kao jedinom izvoru ugljenika i formira jasne zone prosvetljenja. Ovaj gubitak sposobnosti rasta u korelaciji je sa hipotezom da *B. subtilis* BPM12 koristi etilen-glikol, nastao hidrolizom BHET, kao izvor ugljenika. Prisustvo blage zone prosvetljenja, čak i bez vidljivog rasta, ukazuje na moguće učešće i drugih enzima u razgradnji PET monomera i oligomera ali da Bpm12CE svakako igra ključnu ulogu u ovom procesu. Dodatna potvrda dobijena je transformisanjem *B. subtilis* 168 koji nije imao sposobnost razgradnje BHET. Nakon transformacije sa plazmidom koji je nosio *bpm12CE* gen, stepen hidrolize BHET ovog soja postao je skoro identičan onom koji je uočen kod *B. subtilis* BPM12. Paralelnom, homolognom ekspresijom istog gena u *B. subtilis* BPM12 došlo je do 1.6 puta povećane hidrolize BHET u prva 24 h (odeljak 3.1. str. 26). Svi ovi rezultati zajedno ukazuju na to da je Bpm12CE enzim odgovoran za razgradnju PET monomera i oligomera i da soj *B. subtilis* BPM12 može biti iskorišćen kao ekspresiona platforma u sistemima za razgradnju PET.

Bacillus je povoljniji ekspresioni sistem za PETaze u poređenju sa klasičnim sistemima kao što je *E. coli* zbog urođene sposobnosti sekrecije i boljeg uvijanja proteina, što rezultira većim prinosom stabilnijih enzima (Souza et al., 2021, Wei et al., 2019). Do sada su uspešno eksprimirana četiri enzima sa PETaznom aktivnošću upotrebom *Bacillus* sojeva. *T. fusca* hidrolaza (TfH) dobijena je korišćenjem *B. megaterium* sa odličnim prinosom na skali od 2 l (Yang et al., 2007). Naknadno, sličan enzim, TfCut2 takođe je dobijen upotrebom *B. subtilis* sa visokom čistoćom, a i njegova aktivnost na PET je potvrđena. TfCut2 eksprimiran u *Bacillus* pokazuje bolju termalnu

stabilnost u poređenju sa *E. coli*, sa tačkom topljenja višom za 4°C usled pravilnijeg uvijanja enzima (Wei et al., 2019). Optimizacija signalnih peptida omogućila je i poboljšanu sekreciju IsPETaze (Huang et al., 2018, Wang et al., 2020) i BhrPETaze (Xi et al., 2021) u različitim *Bacillus* sojevima. S obzirom na to da *B. subtilis* BPM12 poseduje urođenu aktivnost razgradnje PET monomera i oligomera, mogao bi poslužiti kao idealna platforma za eksprimiranje različitih PETaza i drugih pomoćnih enzima kako bi se poboljšali trenutni sistemi za razgradnju PET otpada.

4.3. *Amycolatopsis mediterranei* ISP5501 hidrolizuje uretansku (karbamatnu) vezu i različite vrste poli(uretana)

Poli(uretani) zauzimaju šesto mesto među plastičnim polimerima koji se najviše proizvode na svetskom nivou i njihova proizvodnja je tek nešto manja od PET. Iako u okosnici polimera sadrže veze podložne hidrolizi tj. biološkoj razgradnji, usled velike raznovrsnosti i nedostataka biokatalizatora koji specifično prepoznaju i hidrolizuju uretansku vezu, efikasan sistem za biološku razgradnju PU i dalje nedostaje (Liu et al., 2021a). Najrasprostranjeniji supstrat za procenu razgradnje PU je koloidna disperzija Impranil (Covestro, Nemačka), alifatični poliestarski PU (Zhang et al., 2022). Impranil je našao široku primenu s obzirom da je razgradnju moguće detektovati spektrofotometrijski prateći smanjenje optičke gustine disperzije (Howard et al., 2001). Međutim, Impranil sa sobom nosi i znatne mane. Pre svega nepoznata hemijska struktura Impranila, zaštićena poslovnom tajnom, otežava napore ka identifikovanju specifičnih mehanizama razgradnje ovog polimera. Vrlo je teško napraviti razliku da li je prilikom razgradnje došlo do hidrolize estarskih ili uretanskih veza polimera, a neki istraživači upozoravaju da nivo smanjenja optičke gustine nije uvek u korelaciji sa stepenom razgradnje (Biffinger et al., 2015). Zbog toga, u potrazi za specifičnijim supstratima, istraživači su se okrenuli malim molekulima koji sadrže uretansku vezu. Molekuli obeleženi *p*-toluensulfonamidom i *p*-nitrofenolom uspešno su korišćeni za ispitivanje mehanizma razgradnje uretanske veze (Magnin et al., 2019b, Gamerith et al., 2016). Skorije, dikarbamati niske molekulske mase nastali glikolizom PU pena iskorišćeni su za identifikaciju i karakterizaciju tri enzima, sposobnih da hidrolizuju uretansku vezu, poreklom iz metagenomske biblioteke (Branson et al., 2023). Tako je model jedinjenje PU-7[16] dobijeno reagovanjem toluen-2,4-diizocijanata (najčešćeg diizocijanata korišćenog u proizvodnji PU) i 2-etoksietanola, iskorišćeno za detaljnu karakterizaciju *A. mediterranei* ISP5501 nakon što je ovaj soj pokazao aktivnost na Impranilu (odeljak 3.2. str 42). S obzirom da je dobijen reagovanjem monomera PU, PU-7 predstavlja teoretski intermedijer razgradnje PU i kao takav oslikava realno molekulsko okruženje sa kojim bi enzim koji razgrađuje uretanske veze došao u kontakt.

Bakterijski soj *A. mediterranei* ISP5501 hidrolizovao je obe uretanske veze prisutne u PU-7. Enzimi uključeni u hidrolizu PU su pre svega esteraze, amidaze i proteaze. Dejstvom amidaza i proteaza uretanska veza se raskida tako da nastaju amin i alkohol (Magnin et al., 2020). Prema literaturnim podacima postoje dva predložena mehanizma hidrolize uretanske veze od strane esteraza. Liu i saradnici predlažu da nakon hidrolize uretanske veze dolazi do formiranja

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[16] IUPAC- bis(2-etoksietil) (4-metil-1,3-fenilen)dikarbamat

karbamatne kiseline i alkohola (Liu et al., 2021a), dok Magnin i saradnici tvrde da će produkti razgradnje biti amin i alkohol, uz oslobađanje molekula ugljen-dioksida zbog nestabilnosti karbamatne kiseline (Magnin et al., 2020). Aktivnošću *A. mediterranei* ISP5501 dobijeni su kako amini tako i karbamatne kiseline, što ukazuje na mogućnost da ovaj soj ima više od jednog enzima sa sposobnošću hidrolize uretanske veze. Pored sposobnosti da razgrađuje Impranil i uretansku vezu u malim molekulima *A. mediterranei* ISP5501 razgrađivao je i polietarski PU, za koji ima vrlo malo literaturnih podataka. Razgradnja polietarskih PU prevashodno se vezuje za gljive iz roda *Alternaria*, *Aspergillus*, *Cladosporium* i *Penicillium* (Álvarez-Barragán et al., 2016, Matsumiya et al., 2010). Podaci o bakterijskoj razgradnji se svode na istraživanja koja uključuju kompleksne zajednice mikroorganizama kako bi se razgradili polietarski PU (Cregut et al., 2013).

Pored rezultata iznetih ovde, *Amycolatopsis* sojevi su se pokazali kao obećavajući kandidati u potrazi za biokatalizatorima sposobnim da razgrade plastiku. Istraživanja o mogućnosti primene *Amycolatopsis* vrsta u razgradnji plastike otkrila su nekoliko efikasnih PLA depolimeraza (Pranamuda and Tokiwa, 1999), a analiza filogenetske distribucije enzima za razgradnju plastike pokazala je da najveći broj PLA depolimeraza vodi poreklo od predstavnika roda *Amycolatopsis* (Gambarini et al., 2021). Dodatno, više od 10 enzima sličnih PETazama takođe je identifikovano različitim u vrstama *Amycolatopsis* (Joo et al., 2018), čineći ovaj rod obećavajućim izvorom novih biokatalizatora za razgradnju plastike ali potencijalno i alatkom za proizvodnju jedinjenja sa dodatom vrednošću imajući u vidu da se *A. mediterranei* koristi za proizvodnju rifamicina[17] na industrijskom nivou (Singhvi et al., 2021).

4.3.1. Enzimi uključeni u razgradnju poli(uretana) kod *A. mediterranei* ISP5501

Razgradnja plastičnih polimera kao što su PU prevashodno se odvija u vanćelijskoj sredini usled visoke molekulske mase i hidrofobnosti polimera, u procesu koji uključuje sekretovane enzime. Međutim, neki mikroorganizmi kao što su *C. acidovorans*, *P. capeferrum* i jedan soj *Penicillium* poseduju membranske proteine sposobne da razgrade PU (Puiggené et al., 2022, Magnin et al., 2019a, Akutsu et al., 1998). U slučaju *P. capeferrum* vanćelijske vezikule pokazivale su sposobnost razgradnje PU ukazujući da membranski proteini mogu biti podjednako važni kao i sekretovani. Slično, testiranjem različitih ćelijskih frakcija utvrđeno je da enzimi uključeni u razgradnju PU kod *A. mediterranei* ISP5501 takođe mogu biti membranski. Kako bi se dodatno suzio izbor, proteom *A. mediterranei* ISP5501 upoređen je sa poznatim enzimima aktivnim na PU iz PAZy baze (odeljak 3.2. str. 44).

Među enzimima homolognim onima iz PAZy baze našle su se dve esteraze homologne sa lipazom Tcur_1278 iz *Thermomonospora curvata*. Ovaj enzim pokazao je aktivnost i na PET, Impranilu i na čvrstim termoplastičnim PU. Međutim, slično drugim sojevima, *A. mediterranei* ISP5501 uprkos odličnoj aktivnosti na Impranilu razgradnja čvrstih PU rezultirala je smanjenjem

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[17] [(7*S*,9*E*,11*S*,12*R*,13*S*,14*R*,15*R*,16*R*,17*S*,18*S*,19*E*,21*Z*)-2,15,17,27,29-pentahidroksi-11-metoksi-3,7,12,14 ,16,18,22-heptametil-6,23-diokso-8,30-dioksa-24-azatetraciklo[23.3.1.1^{4,7}.0^{5,28}]triakonta-1(29),2,4,9,19,21, 25,27-oktaen-13-il]acetat

mase od svega nekoliko procenata (Schmidt et al., 2017). Dodatno, aktivnost *A. mediterranei* ISP5501 na PU može se pripisati i enzimu, koji je 100% identičan, AML kutinazi koji razgrađuje PCL i PBS. Iako ovaj enzim nije testiran direktno na PU, PBS korišćen u studiji koja je ispitivala AML kutinazu bio je dodatno umrežen uretanskim vezama, tako da razgradnja ovog polimera može biti usled razgradnje kako estarskih tako i uretanskih veza (Tan et al., 2021). Kao što je već pomenuto najverovatnije je više enzima *A. mediterranei* ISP5501 uključeno u razgradnju PU i rezultati prezentovani u ovoj disertaciji predstavljaju samo prvi korak u identifikovanju svih enzima odgovornih za ovu aktivnost.

4.4. *Streptomyces* sp. PU10 efikasno razgrađuje poliestarsku poli(uretansku) disperziju

Streptomicete su široko prepoznate zbog svoje uloge kao vrednog rezervoara medicinski značajnih jedinjenja, uključujući antibiotike, antifungalna jedinjenja, imunosupresante i lekove protiv raka (Alam et al., 2022), ali se takođe ističu i po raznolikom enzimskom arsenalu koji se može upotrebiti za različite biotehnološke aplikacije (Spasic et al., 2018, Vojnovic et al., 2024). Ovaj spoj enzimske raznovrsnosti i sposobnosti proizvodnje sekundarnih metabolita iskorišćen je za recikliranje papira, tekstila i otpada iz agroindustrije (Cuebas- Irizarry and Grunden, 2023, Kashiwagi et al., 2017). Kao integralni deo zemljišnog mikrobioma, *Streptomyces* sojevi su relativno malo proučavani u kontekstu razgradnje plastike. Većina istraživanja sprovedeno je na razgradnji PE, međutim, ova istraživanja bazirala su se samo na uočenom formiranju biofilma i površinskim promenama PE bez identifikovanja enzima i jasnog mehanizma delovanja (Soud, 2019, El-Shafei et al., 1998, Han et al., 2020). Više uspeha postignuto je u razgradnji PET pronalaskom enzima SM14est iz marinske vrste *Streptomyces* sp. SM14 (Carr et al., 2023) i razgradnji biorazgradivih polimera kao što je PHA gde je identifikovana nova supergrupa PHA depolimeraza (Gangoiti et al., 2012).

Iako su neki sojevi *Streptomyces* pokazali aktivnost na Impranilu u okviru testova na šoljama, temeljnija istraživanja u ovom smeru nisu sprovedena (Rüthi et al., 2023). *Streptomyces* sp. PU10 pokazao se kao izuzetno efikasan u razgradnji poliestarske PU disperzije Impranila (odeljak 3.3. str. 61). Pokazan stepen i brzina razgradnje prevazišli su i najbolje do sada prijavljene mikroorganizme sposobne da razgrade Impranil. *Cladosporium halotolerans* 6UPA1 razgrađivao je 80%, a *Cladosporium* sp. P7 94.4% Impranila za svega 3 dana (Zhang et al., 2022, Liu et al., 2023a). *Embarria clametidis* MFLUCC 14-0652, izdvojena kao najaktivnija od 33 testirana soja, dostizala je 88.84% razgradnje 1% Impranila za dve nedelje (Khruengsai et al., 2022). Prema FTIR rezultatima, najveći deo razgradnje Impranila od strane *Streptomyces* sp. PU10 pripisan je raskidanju estarskih veza dok su uretanske veze hidrolizovane u nešto manjoj meri. Ovakav trend razgradnje primećen je i kod drugih biokatalizatora sposobnih da razgrade Impranil (Zhang et al., 2022). Imajući u vidu visok nivo razgradnje ali i činjenicu da *Streptomyces* sp. PU10 konvertuje Impranil u biomasu, izvršena je proteomska analiza kako bi se identifikovali enzimi uključeni u razgradnju ali i promene u metaboličkim putevima prilikom korišćenja Impranila kao jedinog izvora ugljenika i energije (**Slika 3**).
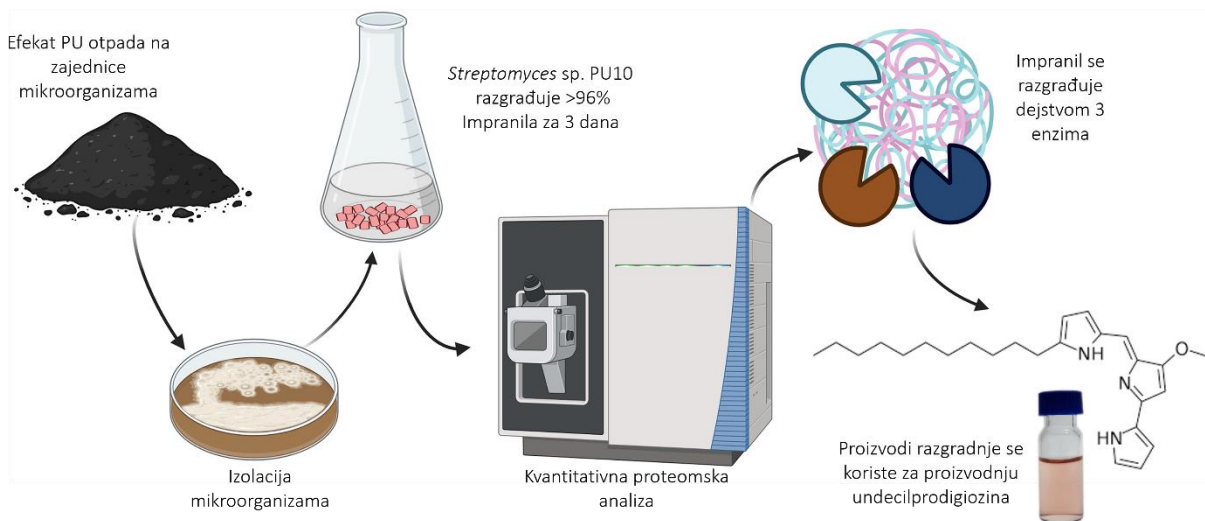
Slika 3. Shematski prikaz toka istraživanja razgradnje PU od strane *Streptomyces* sp. PU10 i najvažnijih dobijenih rezultata.

4.4.1. Biodegradacija poli(uretana) kod *Streptomyces* sp. PU10 se odvija posredstvom Est_PU10, Am_PU10 i Oxr_PU10

Kako PU predstavljaju vrlo heterogenu grupu polimera i u svojoj okosnici sadrže različite tipove hemijskih veza, kao što je istaknuto u odeljku 1.3.4, skorija istraživanja ukazuju na to da je za njihovu efikasnu razgradnju neophodna aktivnost više različitih enzima. Stoga, poseban akcenat stavljen je na esteraze, amidaze ali i oksidoreduktaze iz *Streptomyces* sp. PU10.

Upotreba Impranila kao izvora ugljenika dovela je do povećanja ekspresije hidrolaza, oksidoreduktaza i proteaza - fenomen koji je takođe uočen kod *Fusarium* sp. IA2 i *F. oxysporum* BPOP18 sposobnih da razgrađuju PU (Taxeidis et al., 2023). Predloženi mehanizam razgradnje PU od strane *Streptomyces* sp. PU10 uključuje aktivnost amidaze, esteraze i oksidaze (odeljak 3.3. str. 63). Najveći deo razgradnje najverovatnije se odvija dejstvom konstitutivno eksprimirane esteraze Est_PU10 koja je srodna sa PETazama visoke aktivnosti. Est_PU10 je bliski homolog PHL1, PHL2, PET40 i Enzimu 607 otkrivenih pretraživanjem metagenomskih biblioteka, a koje pokazuju izuzetnu termostabilnost (Sonnendecker et al., 2022, Erickson et al., 2022, Zhang et al., 2023). Povećana stabilnost ezima srodnih sa Est_PU10 može se takođe dovesti u vezu sa činjenicom da *Streptomyces* sp. PU10 razgrađuje Impranil i na temperaturama preko 40°C. Mali molekuli koji sadrže uretansku vezu, oslobođeni dejstvom Est_PU10, dalje indukuju ekspresiju amidaze Am_PU10 za koju je pretpostavljeno da dalje razgrađuje ove molekule do intermedijera koji mogu biti inkorporirani u primarni metabolizam. Ova pretpostavka zasnovana je na homologiji sa uretanazama UMG-SP-1, UMG-SP-2 i UMG-SP-3, enzimima sposobnim da hidrolizuju uretansku vezu u jedinjenjima niske molekulske mase asociranim sa nepotpunom hemijskom razgradnjom PU (Branson et al., 2023). Razgradnja malih molekula koji sadrže uretansku vezu

potencijalno je dodatno potpomognuta dvema hidrolazama, čija je ekspresija takođe bila povećana, a koje su srodne sa enzimom iz *Microbacterium hydrocarbonoxydans* sposobnim da razgrađuje etil karbamat (Kang et al., 2021). Finalno, kao i u primeru *B. velenzis* (Gui et al., 2023), enzim sa najvećom promenom ekspresije bila je oksidaza Oxr_PU10. Oxr_PU10 je daleki homolog Oxr-1 enzima i najverovatnije doprinosi razgradnji Impranila do sada nepoznatim mehanizmom. Iako je u poslednjih nekoliko godina sve više istraživanja fokusirano na upotrebu oksidoreduktaza u razgradnji PU, tačan mehanizam aktivnosti i dalje nije poznat, a mali broj do sada okarakterisanih enzima ograničava upotrebu bioinformatičkih alatki u pronalaženju novih oksidoreduktaza za razgradnju PU.

4.4.2. *Streptomyces* sp. PU10 inkorporira intermedijere razgradnje poli(uretana) u primarni metabolizam i kanališe ih ka sintezi poliketida

Napori ka razumevanju puteva uključenih u inkorporaciju produkata razgradnje plastičnih polimera u metabolizam umnogome zavise od vrste plastike. Polimeri sa strogo definisanom strukturom kao što su PS i PET mnogo su bolje izučeni od PU koji mogu biti sastavljeni od mnoštva različitih gradivnih jedinica. Metabolički put razgradnje stirena[18], monomera PS, poznat je skoro tri decenije (Mooney et al., 2006, Hartmans et al., 1990). Metabolički put razgradnje tereftalne kiseline takođe je poznat uključujući i proteine odgovorne za njen transmembranski transport (Yastrebova et al., 2022, Pardo et al., 2020). Dodatno, i putevi razgradnje 1,4 butandiola, adipinske kiseline i 2,4-dimaminotoluena, nekih od najčešćih produkata razgradnje PU, su otkriveni (put razgradnje 2,4 toluendiamina nije u potpunosti potvrđen) (Li et al., 2020, Parke et al., 2001, Espinosa et al., 2020). Međutim, usled kompleksne strukture PU velika većina produkata razgradnje, među kojima je i jedan od najčešćih produkata- 4,4' metilendianilin, se metaboliše za sada nepoznatim putevima (Liu et al., 2021a).

Povećana ekspresija acetil-CoA sintetaze FadD, enzima zaduženog za započinjanje katabolizma masnih kiselina, kao i drugih enzima iz ovog puta ukazuju na to da se produkti razgradnje Impranila inkorporiraju u metabolizam *Streptomyces* sp. PU10 preko puta katabolizma masnih kiselina (odeljak 3.3. str. 64). Analizom transkriptoma ovaj metabolički put takođe je predložen kao glavni put katabolizma produkata razgradnje Impranila i kod *C. halotolerans* 6UPA1 (Zhang et al., 2022). FadD se odlikuje vrlo širokom supstratnom specifičnošću, kako u *Streptomyces* sojevima tako i u drugim bakterijama, omogućavajući metabolizam alifatičnih i aromatičnih supstrata (Hume et al., 2009). Pored toga, povećana ekspresija FadD povezana je i sa efikasnom proizvodnjom sekundarnih metabolita kod drugih sojeva *Streptomyces* (Banchio and Gramajo, 2002). Acetil-CoA nastao u katabolizmu masnih kiselina dalje postaje supstrat za acetil-CoA karboksilazu BccA i biva transformisan u malonil-CoA. Malonil-CoA i BccA su ključni za premošćavanje jaza između primarnog i sekundarnog metabolizma (Laakel et al., 1994) i identifikovani su kao veza između katabolizma masnih kiselina i biosinteze poliketida (Summers et al., 1995). Povećavanjem ekspresije acetil-CoA karboksilaze i obezbeđivanjem veće količine

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[18] IUPAC- etenilbenzen

malonil-CoA metaboličkim inženjerstvom uspešno je povećan prinos proizvodnje antibiotika kod nekih *Streptomyces* sojeva (Liao et al., 2022, Zabala et al., 2016). Svi gore navedeni procesi ukazuju na to da se proizvodi razgradnje Impranila integrišu u centralni metabolizam *Streptomyces* sp. PU10 i usmeravaju ka proizvodnji poliketida, primarno ka proizvodnji undecilprodigiozina[19]. Poliketida sa karakterističnom tripirolnom strukturom i antimikrobnim, antikancer, antioksidativnim i UV protektivnim svojstvima (Stankovic et al., 2012, Ho et al., 2007). Povećana proizvodnja undecilprodigiozina takođe je uočena kod *S. coelicolor* M145 izloženog česticama nanoplastike PLA, međutim, istraživanja usmerena ka otkrivanju metaboličke osnove ovog odgovora nisu sprovedena (Liu et al., 2023b). Nizak prinos undecilprodigiozina soja *Streptomyces* sp. PU10 može potencijalno objasniti niskom ekspresijom tioesteraze RedJ u ovom soju, s obzirom da aberantna ekspresija ovog proteina dovodi do smanjene sinteze različitih prodigionina (Whicher et al., 2011).

Pored konverzije produkata razgradnje Impranila u poliketide, na osnovu aktivnih biosintetskih puteva, postoje indikacije da *Streptomyces* sp. PU10 može proizvoditi i terpenoide kao što je izorenieraten[20] koji služi kao zaštita od UV zračenja (Wagener et al., 2012) i slabo izučene neribozomalno sintetisane peptide ashimid A[21] i B[22] (Shi et al., 2019). Na osnovu rezultata predstavljenih ovde može se zaključiti da streptomicete predstavljati rezervoar biokatalizatora za razgradnju PU, vredan daljeg istraživanja. Dodatno, ove bakterije poseduju očigledan potencijal za valorizaciju produkata razgradnje PU, bilo iz biološke ili hemijske razgradnje, u jedinjenja sa dodatom vrednošću kao što su poliketidi i niz drugih biološki aktivnih jedinjenja koje proizvode predstavnici ovog roda.

4.5. Biorazgradivi skrob/poli(vinil-alkohol) filmovi mogu se konvertovati u polihidroksibutirat i undecilprodigiozin

Bioplastika može zameniti konvencionalne plastične materijale u mnogim industrijama i proizvodima pružajući rešenja za različite probleme koji se vezuju za akumulaciju plastičnog otpada. Polimeri na bazi termoplastičnog skroba su održiva i biorazgradiva alternativa, a mogu se dobiti iz obnovljivih izvora kao što su kukuruz i krompir (Rommi et al., 2016, Fahrngruber et al., 2019). Važno je istaći da se mehaničke karakteristike polimera na bazi skroba mogu znatno poboljšati dodavanjem aditiva kao što su glicerol[23], sorbitol[24] i urea ili mešanjem sa drugim biorazgradivim polimerima PCL, PLA ili PVA (Tian et al., 2017, Jeremić et al., 2020). Međutim, ukoliko želimo da nove generacije polimera zaista otklone sve nedostatke konvencionalnih

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[19] IUPAC- (2*Z*,5*Z*)-3-metoksi-5-pirol-2-ilidin-2-[(5-undecil-1*H*-pirol-2-il)metilidin]pirol
[20] IUPAC- 1,2,4-trimetil-3-[(1*E*,3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*,17*E*)-3,7,12,16-tetrametil-18-(2,3,6-trimetilfenil)oktadeka-1,3,5,7,9,11,13,15,17-nonaenil]benzen
[21] IUPAC- [(6*S*,8a*R*)-8a-(hidroksimetil)-1-metil-2,5,8-triokso-6,7-dihidro-3*H*-imidazo[1,2-a]pirazin-6-il]metil 2-hidroksi-2-(hidroksimetil)-3-okso-4*H*-1,4-benyoksazin-5-karboksilat
[22] IUPAC- [(6*S*,8a*S*)-8a-(hlorometil)-1-metil-2,5,8-triokso-6,7-dihidro-3*H*-imidazo[1,2-a]pirazin-6-il]metil 2-hidroksi-2-(hidroksimetil)-3-okso-4*H*-1,4-benzoksazin-5-karboksilat
[23] IUPAC- propan-1,2,3,-triol
[24] IUPAC- (2*S*,3*R*,4*R*,5*R*)-heksan-1,2,3,4,5,6-heksol

plastika neophodno je obezbediti sistem koji će biti ne samo ekološki već i ekonomski održiv kroz valorizaciju ovih polimera u jedinjenja sa dodatom vrednošću.

Skrob, kao prirodni polimer, poseduje urođenu biorazgradivost tako da se u uslovima kompostiranja filmovi od termoplastičnog skroba razgrađuju za 30 do 84 dana (Kliem et al., 2020, Torres et al., 2011). Sa druge strane, biorazgradivost PVA je takođe dokazana i dobro dokumentovana. *Bacillus* spp, *Pseudomonas* spp, i različiti sojevi kvasaca pokazali su se kao vrlo efikasni u razgradnji PVA *in vivo* (Mori et al., 1996, Liu et al., 2019, Chiellini et al., 2003). Iako kompostiranje predstavlja efikasan način zbrinjavanja biorazgradivog otpada, na ovaj način ugljenik koji može biti iskorišćen za proizvodnju vrednih jedinjenja biva efektivno izgubljen. Soj *Ralstonia eutropha* H16 uspešno je preveo skrob/PVA filmove korišćene u ovom istraživanju u PHB (odeljak 3.4. str. 80). Ranije je proizvodnja PHB iz skroba uspešno je postignuta primenom *Azotobacter chroococcum*, *Heloferax mediterranei* ali i drugim sojevima *R. eutropha* (Kim, 2000, Haas et al., 2008), međutim proizvodnja PHB iz PVA i polimera koji predstavljaju mešavinu skroba i drugih biorazgradivih polimera nije do sada prijavljena. Relativno nizak prinos PHB iz skrob/PVA filmova može se objasniti nedostatkom amilaza i glukoamilaza, enzima neophodnih za razgradnju skroba (Volodina et al., 2016). Ubuduće nizak prinos ovog procesa može se prevazići saharifikacijom skroba kao što je pokazano u slučaju *R. eutropha* koja je proizvodila visoke koncentracije PHB iz saharifikovanog skroba iz krompira (Haas et al., 2008) ili upotrebom tehnika genetičkog inženjerstva. Tako je *Corynebacterium glutamicum* koji prirodno proizvodi PHB modifikovan da na površini eksprimira α–amilazu kako bi se postigla efikasna proizvodnja PHB koristeći skrob kao jedini izvor ugljenika (Volodina et al., 2016).

Konverzija skrob/PVA filmova u PHB predstavlja značajan porast u vrednosti koji se može dobiti od ovih filmova obzirom da je tržišna vrednost 1 kg skrob/PVA granula 3-4 EUR naspram 10-24 EUR koliko vredi 1 kg PHB (Manikandan et al., 2021). Alternativno, upotrebom *Streptomyces* sp. JS520 skrob/PVA filmovi mogu se pretvoriti u undecilprodigiozin čija je vrednost, prema podacima proizvođača, ~360 EUR/mg (odeljak 3.4. str. 79). Pored jasnih ekonomskih prednosti, kao što je već pomenuto, undecilprodigiozin se karakteriše nizom korisnih bioloških aktivnosti (Ho et al., 2007). Proizvodnja undecilprodigiozina uspešno je postignuta upotrebom različitih prostih šećera ali i skroba i celuloze kao izvora ugljenika kod *S. coelicolor* (Luti, 2018). Najviši do sada prijavljeni prinos undecilprodigiozina iz *Streptomyces* sp. JS520 je 12 puta veći od prinosa dobijenog upotrebom skrob/PVA kao jedinog izvora ugljenika (Stankovic et al., 2012). Međutim, važno je napomenuti da je takav prinos dobijen upotrebom optimizovanih kako podloga za gajenje, tako i samog procesa proizvodnje, što ukazuje na mogućnost znatnog povećanja prinosa ovog metabolita.. Sa druge strane, istraživanja o upotrebi otpada kao izvora ugljenika za proizvodnju undecilprodigiozina su vrlo limitirana i odnose se samo na otpad iz agroindustrije (Luti and Yonis, 2014). Valorizacija bioplastičnih polimera kroz prevođenje u biološki aktivna jedinjenja predstavlja važan stepenik u razvijanju održivih tehnologija koje za cilj imaju smanjenje plastičnog otpada i integrisanje bioplastike u široku upotrebu. A upotreba otpadnih tokova u biotehnološkim procesima je ujedno i korak ka smanjenju cene proizvodnje terapeutski značajnih jedinjenja.

5. ZAKLJUČCI

Uzevši u obzir opšte i specifične ciljeve ove doktorske disertacije, a na osnovu dobijenih rezultata izvedeni su sledeći zaključci:

Pretraživanjem kolekcije mikroorganizama Grupe za eko-biotehnologiju i razvoj lekova (IMGGI) poreklom iz različitih zagađenih i nezagađenih staništa ustanovljeno je sledeće:

1. *B. subtilis* BPM12 se može koristiti u biotehnološkoj razgradnji PET s obzirom da ima aktivnost na monomerima i oligomerima koji ulaze u sastav PET, izaziva morfološke promene na PET filmovima i vezuje se za površinu recikliranog i nerecikliranog PET;
2. Karboksilesteraza nazvana Bpm12CE ima ključnu ulogu u razgradnji PET monomera i oligomera, čineći *B. subtilis* BPM12 pogodnom platformom za ekspresiju različitih PETaza;
3. *A. mediterranei* ISP5501 se može koristiti u biotehnološkoj razgradnji PU s obzirom da specifično hidrolizuje uretansku (karbamatnu) vezu u jedinjenjima niske molekulske mase i razgrađuje kako poliestarske tako i polietarske PU;
4. *Streptomyces* sp. PU10 se može koristiti u biotehnološkoj razgradnji PU s obzirom da se pokazao kao jedan od najpotentnijih sojeva za razgradnju poliestarske PU disperzije Impranila;
5. Razgradnja PU od strane *Streptomyces* sp. PU10 uključuje sinergističko dejstvo više enzima: esteraze Est_PU10 je zadužena za najveći deo razgradnje kroz hidrolizu estarskih veza, amidaze Am_PU10 hidrolizuje uretansku (karbamatnu) vezu u intermedijerima razgradnje, dok oksidaze Oxr_PU10 koja doprinosi razgradnji do sada još nepoznatim mehanizmom;
6. Prilikom razgradnje PU, *Streptomyces* sp. PU10 inkorporira produkte razgradnje u primarni metabolizam kroz put katabolizma masnih kiselina, koje zatim kanališe ka biosintezi poliketida, među kojima je i undecilprodigiozin;
7. Upotrebom *R. eutropha* H16 i *Streptomyces* sp. JS520 biorazgradivi skrob/PVA filmovi mogu se prevesti u PHB i undecilprodigiozin, pružajući način valorizacije otpadne bioplastike u jedinjenja sa dodatom vrednošću.

6. LITERATURA

AKUTSU, Y., NAKAJIMA-KAMBE, T., NOMURA, N. & NAKAHARA, T. 1998. Purification and properties of a polyester polyurethane-degrading enzyme from *Comamonas acidovorans* TB-35. *Appl. Environ. Microbiol.,* 64**,** 62-67.

ALAM, K., MAZUMDER, A., SIKDAR, S., ZHAO, Y.-M., HAO, J., SONG, C., WANG, Y., SARKAR, R., ISLAM, S. & ZHANG, Y. 2022. *Streptomyces*: The biofactory of secondary metabolites. *Frontiers in Microbiology,* 13**,** 968053.

ALI, S. S., ELSAMAHY, T., KOUTRA, E., KORNAROS, M., EL-SHEEKH, M., ABDELKARIM, E. A., ZHU, D. & SUN, J. 2021. Degradation of conventional plastic wastes in the environment: A review on current status of knowledge and future perspectives of disposal. *Science of The Total Environment,* 771**,** 144719.

ALONSO, J. A., AGUADO, J. & SERRANO, D. P. 1999. *Feedstock recycling of plastic wastes*, Royal society of chemistry.

ÁLVAREZ-BARRAGÁN, J., DOMÍNGUEZ-MALFAVÓN, L., VARGAS-SUÁREZ, M., GONZÁLEZ-HERNÁNDEZ, R., AGUILAR-OSORIO, G. & LOZA-TAVERA, H. 2016. Biodegradative activities of selected environmental fungi on a polyester polyurethane varnish and polyether polyurethane foams. *Appl. Environ. Microbiol.,* 82**,** 5225-5235.

AMOBONYE, A., BHAGWAT, P., SINGH, S. & PILLAI, S. 2021. Plastic biodegradation: Frontline microbes and their enzymes. *Science of the Total Environment,* 759**,** 143536.

ANIK, A. H., HOSSAIN, S., ALAM, M., SULTAN, M. B., HASNINE, M. T. & RAHMAN, M. M. 2021. Microplastics pollution: A comprehensive review on the sources, fates, effects, and potential remediation. *Environmental Nanotechnology, Monitoring & Management,* 16**,** 100530.

ARUTCHELVI, J., SUDHAKAR, M., ARKATKAR, A., DOBLE, M., BHADURI, S. & UPPARA, P. V. 2008. Biodegradation of polyethylene and polypropylene.

AUTA, H. S., EMENIKE, C. U., JAYANTHI, B. & FAUZIAH, S. H. 2018. Growth kinetics and biodeterioration of polypropylene microplastics by *Bacillus* sp. and *Rhodococcus* sp. isolated from mangrove sediment. *Marine pollution bulletin,* 127**,** 15-21.

BANCHIO, C. & GRAMAJO, H. 2002. A stationary-phase acyl-coenzyme A synthetase of *Streptomyces* coelicolor A3 (2) is necessary for the normal onset of antibiotic production. *Applied and environmental microbiology,* 68**,** 4240-4246.

BAO, T., QIAN, Y., XIN, Y., COLLINS, J. J. & LU, T. 2023. Engineering microbial division of labor for plastic upcycling. *Nature communications,* 14**,** 5712.

BARTH, M., HONAK, A., OESER, T., WEI, R., BELISÁRIO-FERRARI, M. R., THEN, J., SCHMIDT, J. & ZIMMERMANN, W. 2016. A dual enzyme system composed of a polyester hydrolase and a carboxylesterase enhances the biocatalytic degradation of polyethylene terephthalate films. *Biotechnology Journal,* 11**,** 1082-1087.

BENTHAM, R., MORTON, L. & ALLEN, N. 1987. Rapid assessment of the microbial deterioration of polyurethanes. *International biodeterioration,* 23**,** 377-386.

BERGMANN, M., ALMROTH, B. C., BRANDER, S. M., DEY, T., GREEN, D. S., GUNDOGDU, S., KRIEGER, A., WAGNER, M. & WALKER, T. R. 2022. A global plastic treaty must cap production. *Science,* 376**,** 469-470.

BHANOT, V., GUPTA, S. & PANWAR, J. 2024. Phylloplane fungus *Curvularia dactyloctenicola* VJP08 effectively degrades commercially available PS product. *Journal of Environmental Management,* 351**,** 119920.

BHAVSAR, P., BHAVE, M. & WEBB, H. K. 2023. Effective multi-stage biodegradation of commercial bulk polyurethane by *Clonostachys* and *Purpureocillium* spp. *Science of The Total Environment***,** 168329.

BIFFINGER, J. C., BARLOW, D. E., COCKRELL, A. L., CUSICK, K. D., HERVEY, W. J., FITZGERALD, L. A., NADEAU, L. J., HUNG, C. S., CROOKES-GOODSON, W. J. & RUSSELL JR, J. N. 2015. The applicability of Impranil® DLN for gauging the biodegradation of polyurethanes. *Polymer Degradation and Stability,* 120**,** 178-185.

BLANK, L. M., NARANCIC, T., MAMPEL, J., TISO, T. & O'CONNOR, K. 2020. Biotechnological upcycling of plastic waste and other non-conventional feedstocks in a circular economy. *Current opinion in biotechnology,* 62**,** 212-219.

BOLLINGER, A., THIES, S., KNIEPS-GRÜNHAGEN, E., GERTZEN, C., KOBUS, S., HÖPPNER, A., FERRER, M., GOHLKE, H., SMITS, S. H. & JAEGER, K.-E. 2020. A novel polyester hydrolase from the marine bacterium *Pseudomonas* aestusnigri–structural and functional insights. *Frontiers in microbiology,* 11**,** 114.

BONIFER, K. S., WEN, X., HASIM, S., PHILLIPS, E. K., DUNLAP, R. N., GANN, E. R., DEBRUYN, J. M. & REYNOLDS, T. B. 2019. *Bacillus pumilu*s B12 degrades polylactic acid and degradation is affected by changing nutrient conditions. *Frontiers in microbiology,* 10**,** 2548.

BORNSCHEUER, U. T. 2018. The fourth wave of biocatalysis is approaching. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences,* 376**,** 20170063.

BORNSCHEUER, U. T., HUISMAN, G., KAZLAUSKAS, R., LUTZ, S., MOORE, J. & ROBINS, K. 2012. Engineering the third wave of biocatalysis. *Nature,* 485**,** 185-194.

BRANSON, Y., SÖLTL, S., BUCHMANN, C., WEI, R., SCHAFFERT, L., BADENHORST, C. P., REISKY, L., JÄGER, G. & BORNSCHEUER, U. T. 2023. Urethanases for the enzymatic hydrolysis of low molecular weight carbamates and the recycling of polyurethanes. *Angewandte Chemie International Edition,* 62**,** e202216220.

BUCHHOLZ, P., ZHANG, H., PEREZ-GARCIA, P., NOVER, L.-L., CHOW, J., STREIT, W. R. & PLEISS, J. 2022. Plastics degradation by hydrolytic enzymes: the Plastics-Active Enzymes Database-PAZy. *Proteins: Structure, Function, and Bioinformatics* 90 (7)**,** 1443-1456.

CARR, C. M., CLARKE, D. J. & DOBSON, A. D. 2020. Microbial polyethylene terephthalate hydrolases: current and future perspectives. *Frontiers in Microbiology,* 11**,** 571265.

CARR, C. M., KELLER, M. B., PAUL, B., SCHUBERT, S. W., CLAUSEN, K. S., JENSEN, K., CLARKE, D. J., WESTH, P. & DOBSON, A. D. 2023. Purification and biochemical characterization of SM14est, a PET-hydrolyzing enzyme from the marine sponge-derived *Streptomyces* sp. SM14. *Frontiers in Microbiology,* 14**,** 1170880.

CARUSO, G. 2019. Microplastics as vectors of contaminants. *Marine pollution bulletin,* 146**,** 921-924.

CHAMAS, A., MOON, H., ZHENG, J., QIU, Y., TABASSUM, T., JANG, J. H., ABU-OMAR, M., SCOTT, S. L. & SUH, S. 2020. Degradation rates of plastics in the environment. *ACS Sustainable Chemistry & Engineering,* 8**,** 3494-3511.

CHARNOCK, C. 2021. Norwegian soils and waters contain mesophilic, plastic-degrading bacteria. *Microorganisms,* 9**,** 94.

CHEN, C.-C., DAI, L., MA, L. & GUO, R.-T. 2020. Enzymatic degradation of plant biomass and synthetic polymers. *Nature Reviews Chemistry,* 4**,** 114-126.

CHEN, G.-Q. 2009. A microbial polyhydroxyalkanoates (PHA) based bio-and materials industry. *Chemical society reviews,* 38**,** 2434-2446.

CHEN, Z., DUAN, R., XIAO, Y., WEI, Y., ZHANG, H., SUN, X., WANG, S., CHENG, Y., WANG, X. & TONG, S. 2022. Biodegradation of highly crystallized poly (ethylene terephthalate) through cell surface codisplay of bacterial PETase and hydrophobin. *Nature Communications,* 13**,** 1-17.

CHIELLINI, E., CORTI, A., D'ANTONE, S. & SOLARO, R. 2003. Biodegradation of poly (vinyl alcohol) based materials. *Progress in Polymer science,* 28**,** 963-1014.

CHOW, J., PEREZ-GARCIA, P., DIERKES, R. & STREIT, W. R. 2022. Microbial enzymes will offer limited solutions to the global plastic pollution crisis. *Microbial Biotechnology*.

CIRIC, M., ŠARABA, V., BUDIN, C., DE BOER, T. & NIKODINOVIC-RUNIC, J. 2024. Polyurethane-Degrading Potential of Alkaline Groundwater Bacteria. *Microbial Ecology,* 87**,** 21.

CONNOR, A., LAMB, J. V., DELFERRO, M., KOFFAS, M. & ZHA, R. H. 2023. Two-step conversion of polyethylene into recombinant proteins using a microbial platform. *Microbial Cell Factories,* 22**,** 214.

CREGUT, M., BEDAS, M., DURAND, M.-J. & THOUAND, G. 2013. New insights into polyurethane biodegradation and realistic prospects for the development of a sustainable waste recycling process. *Biotechnology advances,* 31**,** 1634-1647.

CUEBAS-IRIZARRY, M. F. & GRUNDEN, A. M. 2023. *Streptomyces* spp. as biocatalyst sources in pulp and paper and textile industries: Biodegradation, bioconversion and valorization of waste. *Microbial Biotechnology*.

DA COSTA, A. M., DE OLIVEIRA LOPES, V. R., VIDAL, L., NICAUD, J.-M., DE CASTRO, A. M. & COELHO, M. A. Z. 2020. Poly (ethylene terephthalate)(PET) degradation by *Yarrowia lipolytica*: Investigations on cell growth, enzyme production and monomers consumption. *Process Biochemistry,* 95**,** 81-90.

DEGLI ESPOSTI, M., MORSELLI, D., FAVA, F., BERTIN, L., CAVANI, F., VIAGGI, D. & FABBRI, P. 2021. The role of biotechnology in the transition from plastics to bioplastics: An opportunity to reconnect global growth with sustainability. *FEBS open bio,* 11**,** 967-983.

DJAPOVIC, M., MILIVOJEVIC, D., ILIC-TOMIC, T., LJEŠEVIĆ, M., NIKOLAIVITS, E., TOPAKAS, E., MASLAK, V. & NIKODINOVIC-RUNIC, J. 2021. Synthesis and characterization of polyethylene terephthalate (PET) precursors and potential degradation products: Toxicity study and application in discovery of novel PETases. *Chemosphere,* 275**,** 130005.

EL-SHAFEI, H. A., ABD EL-NASSER, N. H., KANSOH, A. L. & ALI, A. M. 1998. Biodegradation of disposable polyethylene by fungi and Streptomyces species. *Polymer degradation and stability,* 62**,** 361-365.

ERICKSON, E., GADO, J. E., AVILÁN, L., BRATTI, F., BRIZENDINE, R. K., COX, P. A., GILL, R., GRAHAM, R., KIM, D.-J. & KÖNIG, G. 2022. Sourcing thermotolerant poly (ethylene terephthalate) hydrolase scaffolds from natural diversity. *Nature Communications,* 13**,** 7850.

ESPINOSA, M. J. C., BLANCO, A. C., SCHMIDGALL, T., ATANASOFF-KARDJALIEFF, A. K., KAPPELMEYER, U., TISCHLER, D., PIEPER, D. H., HEIPIEPER, H. J. & EBERLEIN, C. 2020. Toward biorecycling: Isolation of a soil bacterium that grows on a polyurethane oligomer and monomer. *Frontiers in microbiology,* 11**,** 404.

FAHRNGRUBER, B., EICHELTER, J., ERHÄUSL, S., SEIDL, B., WIMMER, R. & MUNDIGLER, N. 2019. Potato-fiber modified thermoplastic starch: Effects of fiber content on material properties and compound characteristics. *European Polymer Journal,* 111**,** 170-177.

FIELDS, R., RODRIGUEZ, F. & FINN, R. 1974. Microbial degradation of polyesters: Polycaprolactone degraded by *P. pullulans*. *Journal of Applied Polymer Science,* 18**,** 3571-3579.

FURUKAWA, M., KAWAKAMI, N., ODA, K. & MIYAMOTO, K. 2018. Acceleration of enzymatic degradation of poly (ethylene terephthalate) by surface coating with anionic surfactants. *ChemSusChem,* 11**,** 4018-4025.

FURUKAWA, M., KAWAKAMI, N., TOMIZAWA, A. & MIYAMOTO, K. 2019a. Efficient Degradation of Poly (ethylene terephthalate) with *Thermobifida fusca* Cutinase Exhibiting Improved Catalytic Activity Generated using Mutagenesis and Additive-based Approaches. *Scientific reports,* 9**,** 1-9.

FURUKAWA, M., KAWAKAMI, N., TOMIZAWA, A. & MIYAMOTO, K. 2019b. Efficient degradation of poly (ethylene terephthalate) with *Thermobifida fusca* cutinase exhibiting improved catalytic activity generated using mutagenesis and additive-based approaches. *Scientific reports,* 9**,** 16038.

GAMBARINI, V., PANTOS, O., KINGSBURY, J. M., WEAVER, L., HANDLEY, K. M. & LEAR, G. 2021. Phylogenetic Distribution of Plastic-Degrading Microorganisms. *Msystems,* 6**,** e01112-20.

GAMERITH, C., ACERO, E. H., PELLIS, A., ORTNER, A., VIELNASCHER, R., LUSCHNIG, D., ZARTL, B., HAERNVALL, K., ZITZENBACHER, S. & STROHMEIER, G. 2016. Improving enzymatic polyurethane hydrolysis by tuning enzyme sorption. *Polymer degradation and stability,* 132**,** 69-77.

GANGOITI, J., SANTOS, M., PRIETO, M. A., DE LA MATA, I., SERRA, J. L. & LLAMA, M. J. 2012. Characterization of a novel subgroup of extracellular medium-chain-length polyhydroxyalkanoate depolymerases from actinobacteria. *Applied and environmental microbiology,* 78**,** 7229-7237.

GANGOLA, S., SHARMA, A., BHATT, P., KHATI, P. & CHAUDHARY, P. 2018. Presence of esterase and laccase in *Bacillus subtilis* facilitates biodegradation and detoxification of cypermethrin. *Scientific reports,* 8**,** 12755.

GENTA, M., IWAYA, T., SASAKI, M., GOTO, M. & HIROSE, T. 2005. Depolymerization mechanism of poly (ethylene terephthalate) in supercritical methanol. *Industrial & engineering chemistry research,* 44**,** 3894-3900.

GEYER, R., JAMBECK, J. R. & LAW, K. L. 2017. Production, use, and fate of all plastics ever made. *Science advances,* 3**,** e1700782.

GUI, Z., LIU, G., LIU, X., CAI, R., LIU, R. & SUN, C. 2023. A Deep-Sea Bacterium Is Capable of Degrading Polyurethane. *Microbiology Spectrum***,** e00073-23.

GUZIK, M. W., KENNY, S. T., DUANE, G. F., CASEY, E., WOODS, T., BABU, R. P., NIKODINOVIC-RUNIC, J., MURRAY, M. & O'CONNOR, K. E. 2014. Conversion of post consumer polyethylene to the biodegradable polymer polyhydroxyalkanoate. *Applied microbiology and biotechnology,* 98**,** 4223-4232.

HAAS, R., JIN, B. & ZEPF, F. T. 2008. Production of poly (3-hydroxybutyrate) from waste potato starch. *Bioscience, biotechnology, and biochemistry,* 72**,** 253-256.

HAN, Y.-N., WEI, M., HAN, F., FANG, C., WANG, D., ZHONG, Y.-J., GUO, C.-L., SHI, X.-Y., XIE, Z.-K. & LI, F.-M. 2020. Greater biofilm formation and increased biodegradation of polyethylene film by a microbial consortium of *Arthrobacter* sp. and *Streptomyces* sp. *Microorganisms,* 8**,** 1979.

HANIM, C. 2017. Effect of pH and Temperature on Bacillus subtilis FNCC 0059 Oxalate Decarboxylase Activity. *Pakistan Journal of Biological Sciences: PJBS,* 20**,** 436-441.

HARTMANS, S., VAN DER WERF, M. & DE BONT, J. 1990. Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase. *Applied and Environmental Microbiology,* 56**,** 1347-1351.

HERRERA, D. A. G., MOJICEVIC, M., PANTELIC, B., JOSHI, A., COLLINS, C., BATISTA, M., TORRES, C., FREITAS, F., MURRAY, P. & NIKODINOVIC-RUNIC, J. 2023. Exploring Microorganisms from Plastic-Polluted Sites: Unveiling Plastic Degradation and PHA Production Potential. *Microorganisms,* 11**,** 2914.

HO, T.-F., MA, C.-J., LU, C.-H., TSAI, Y.-T., WEI, Y.-H., CHANG, J.-S., LAI, J.-K., CHEUH, P.-J., YEH, C.-T. & TANG, P.-C. 2007. Undecylprodigiosin selectively induces apoptosis in human breast carcinoma cells independent of p53. *Toxicology and Applied Pharmacology,* 225**,** 318-328.

HONUS, S., KUMAGAI, S., FEDORKO, G., MOLNÁR, V. & YOSHIOKA, T. 2018. Pyrolysis gases produced from individual and mixed PE, PP, PS, PVC, and PET—Part I: Production and physical properties. *Fuel,* 221**,** 346-360.

HORODYTSKA, O., KIRITSIS, D. & FULLANA, A. 2020. Upcycling of printed plastic films: LCA analysis and effects on the circular economy. *Journal of Cleaner Production,* 268**,** 122138.

HOWARD, G., VICKNAIR, J. & MACKIE, R. 2001. Sensitive plate assay for screening and detection of bacterial polyurethanase activity. *Letters in applied microbiology,* 32**,** 211-214.

HOWARD, S. A. & MCCARTHY, R. R. 2023. Modulating biofilm can potentiate activity of novel plastic-degrading enzymes. *npj Biofilms and Microbiomes,* 9**,** 72.

HU, X., THUMARAT, U., ZHANG, X., TANG, M. & KAWAI, F. 2010. Diversity of polyester-degrading bacteria in compost and molecular analysis of a thermoactive esterase from *Thermobifida alba* AHK119. *Applied microbiology and biotechnology,* 87**,** 771-779.

HUANG, X., CAO, L., QIN, Z., LI, S., KONG, W. & LIU, Y. 2018. Tat-independent secretion of polyethylene terephthalate hydrolase PETase in *Bacillus subtilis* 168 mediated by its native signal peptide. *Journal of agricultural and food chemistry,* 66**,** 13217-13227.

HUME, A. R., NIKODINOVIC-RUNIC, J. & O'CONNOR, K. E. 2009. FadD from *Pseudomonas putida* CA-3 is a true long-chain fatty acyl coenzyme A synthetase that activates phenylalkanoic and alkanoic acids. *Journal of bacteriology,* 191**,** 7554-7565.

JABLOUNE, R., KHALIL, M., MOUSSA, I. E. B., SIMAO-BEAUNOIR, A.-M., LERAT, S., BRZEZINSKI, R. & BEAULIEU, C. 2020. Enzymatic degradation of p-nitrophenyl esters, polyethylene terephthalate, cutin, and suberin by Sub1, a suberinase encoded by the plant pathogen *Streptomyces scabies*. *Microbes and environments,* 35**,** ME19086.

JEHANNO, C., ALTY, J. W., ROOSEN, M., DE MEESTER, S., DOVE, A. P., CHEN, E. Y.-X., LEIBFARTH, F. A. & SARDON, H. 2022. Critical advances and future opportunities in upcycling commodity polymers. *Nature,* 603**,** 803-814.

JEREMIĆ, S., MILOVANOVIĆ, J., MOJICEVIĆ, M., ŠKARO BOGOJEVIĆ, S. & NIKODINOVIĆ-RUNIĆ, J. 2020. Understanding bioplastic materials-current state and trends. *Journal of the Serbian Chemical Society,* 85**,** 1507-1538.

JOO, S., CHO, I. J., SEO, H., SON, H. F., SAGONG, H.-Y., SHIN, T. J., CHOI, S. Y., LEE, S. Y. & KIM, K.-J. 2018. Structural insight into molecular mechanism of poly (ethylene terephthalate) degradation. *Nature communications,* 9**,** 1-12.

KANG, T., LIN, J., YANG, L. & WU, M. 2021. Expression, isolation, and identification of an ethanol-resistant ethyl carbamate-degrading amidase from *Agrobacterium tumefaciens* d3. *Journal of bioscience and bioengineering,* 132**,** 220-225.

KASHIWAGI, N., OGINO, C. & KONDO, A. 2017. Production of chemicals and proteins using biomass-derived substrates from a *Streptomyces* host. *Bioresource Technology,* 245**,** 1655-1663.

KAWAI, F., KAWABATA, T. & ODA, M. 2019. Current knowledge on enzymatic PET degradation and its possible application to waste stream management and other fields. *Applied microbiology and biotechnology,* 103**,** 4253-4268.

KAWAI, F., KAWABATA, T. & ODA, M. 2020. Current state and perspectives related to the polyethylene terephthalate hydrolases available for biorecycling. *ACS Sustainable Chemistry & Engineering,* 8**,** 8894-8908.

KENNY, S. T., RUNIC, J. N., KAMINSKY, W., WOODS, T., BABU, R. P., KEELY, C. M., BLAU, W. & O'CONNOR, K. E. 2008. Up-cycling of PET (polyethylene terephthalate) to the biodegradable plastic PHA (polyhydroxyalkanoate). *Environmental science & technology,* 42**,** 7696-7701.

KHRUENGSAI, S., SRIPAHCO, T. & PRIPDEEVECH, P. 2022. Biodegradation of Polyester Polyurethane by Embarria clematidis. *Frontiers in Microbiology,* 13.

KIM, B. S. 2000. Production of poly (3-hydroxybutyrate) from inexpensive substrates. *Enzyme and microbial technology,* 27**,** 774-777.

KIM, H. R., LEE, H. M., JEON, E., YU, H. C., LEE, S., LI, J. & KIM, D.-H. 2019a. Biodegradation of Polystyrene by *Pseudomonas* sp. Isolated from the Gut of Superworms.

KIM, H. T., HEE RYU, M., JUNG, Y. J., LIM, S., SONG, H. M., PARK, J., HWANG, S. Y., LEE, H. S., YEON, Y. J. & SUNG, B. H. 2021. Chemo-Biological Upcycling of Poly (ethylene terephthalate) to Multifunctional Coating Materials. *ChemSusChem,* 14**,** 4251-4259.

KIM, H. T., KIM, J. K., CHA, H. G., KANG, M. J., LEE, H. S., KHANG, T. U., YUN, E. J., LEE, D.-H., SONG, B. K. & PARK, S. J. 2019b. Biological Valorization of Poly (ethylene terephthalate) Monomers for Upcycling Waste PET. *ACS Sustainable Chemistry & Engineering,* 7**,** 19396-19406.

KLEEBERG, I., WELZEL, K., VANDENHEUVEL, J., MÜLLER, R.-J. & DECKWER, W.-D. 2005. Characterization of a new extracellular hydrolase from *Thermobifida fusca* degrading aliphatic– aromatic copolyesters. *Biomacromolecules,* 6**,** 262-270.

KLIEM, S., KREUTZBRUCK, M. & BONTEN, C. 2020. Review on the biological degradation of polymers in various environments. *Materials,* 13**,** 4586.

KOSIOROWSKA, K. E., MORENO, A. D., IGLESIAS, R., LELUK, K. & MIROŃCZUK, A. M. 2022. Production of PETase by engineered *Yarrowia lipolytica* for efficient poly (ethylene terephthalate) biodegradation. *Science of the Total Environment,* 846**,** 157358.

KUMARI, A., BANO, N., BAG, S. K., CHAUDHARY, D. R. & JHA, B. 2021. Transcriptome-Guided Insights Into Plastic Degradation by the Marine Bacterium. *Frontiers in Microbiology***,** 2761.

LAAKEL, M., LEBRIHI, A., KHAOUA, S., SCHNEIDER, F., LEFEBVRE, G. & GERMAIN, P. 1994. A link between primary and secondary metabolism: malonyl-CoA formation in *Streptomyces ambofaciens* growing on ammonium ions or valine. *Microbiology,* 140**,** 1451-1456.

LI, W.-J., NARANCIC, T., KENNY, S. T., NIEHOFF, P.-J., O'CONNOR, K., BLANK, L. M. & WIERCKX, N. 2020. Unraveling 1, 4-butanediol metabolism in *Pseudomonas putida* KT2440. *Frontiers in microbiology,* 11**,** 382.

LIANG, C., GRACIDA-ALVAREZ, U. R., GALLANT, E. T., GILLIS, P. A., MARQUES, Y. A., ABRAMO, G. P., HAWKINS, T. R. & DUNN, J. B. 2021. Material Flows of Polyurethane in the United States. *Environmental Science & Technology,* 55**,** 14215-14224.

LIAO, Z., ZHANG, J., SHI, Y., ZHANG, Y., MA, Z., BECHTHOLD, A. & YU, X. 2022. Improvement of rimocidin biosynthesis by increasing supply of precursor malonyl-CoA via over-expression of acetyl-CoA carboxylase in *Streptomyces rimosus* M527. *Current Microbiology,* 79**,** 174.

LIU, J., HE, J., XUE, R., XU, B., QIAN, X., XIN, F., BLANK, L. M., ZHOU, J., WEI, R. & DONG, W. 2021a. Biodegradation and up-cycling of polyurethanes: Progress, challenges, and prospects. *Biotechnology Advances***,** 107730.

LIU, J., ZENG, Q., LEI, H., XIN, K., XU, A., WEI, R., LI, D., ZHOU, J., DONG, W. & JIANG, M. 2023a. Biodegradation of polyester polyurethane by *Cladosporium* sp. P7: Evaluating its degradation capacity and metabolic pathways. *Journal of Hazardous Materials,* 448**,** 130776.

LIU, L., FOKKINK, R. & KOELMANS, A. A. 2016. Sorption of polycyclic aromatic hydrocarbons to polystyrene nanoplastic. *Environmental toxicology and chemistry,* 35**,** 1650-1655.

LIU, P., ZHANG, T., ZHENG, Y., LI, Q., SU, T. & QI, Q. 2021b. Potential one-step strategy for PET degradation and PHB biosynthesis through co-cultivation of two engineered microorganisms. *Engineering Microbiology,* 1**,** 100003.

LIU, X., AHMAD, S., MA, J., WANG, D. & TANG, J. 2023b. Comparative study on the toxic effects of secondary nanoplastics from biodegradable and conventional plastics on *Streptomyces coelicolor* M145. *Journal of Hazardous Materials,* 460**,** 132343.

LIU, Y., DENG, Y., CHEN, P., DUAN, M., LIN, X. & ZHANG, Y. 2019. Biodegradation analysis of polyvinyl alcohol during the compost burial course. *Journal of basic microbiology,* 59**,** 368-374.

LIU, Z., FANG, Z., ZHENG, N., YANG, K., SUN, Z., LI, S., LI, W., WU, J. & XIE, T. 2023c. Chemical upcycling of commodity thermoset polyurethane foams towards high-performance 3D photo-printing resins. *Nature Chemistry***,** 1-7.

LU, H., DIAZ, D. J., CZARNECKI, N. J., ZHU, C., KIM, W., SHROFF, R., ACOSTA, D. J., ALEXANDER, B. R., COLE, H. O. & ZHANG, Y. 2022. Machine learning-aided engineering of hydrolases for PET depolymerization. *Nature,* 604**,** 662-667.

LUFT, A., BRÖDER, K., KUNKEL, U., SCHULZ, M., DIETRICH, C., BAIER, R., HEININGER, P. & TERNES, T. A. 2017. Nontarget analysis via LC-QTOF-MS to assess the release of organic substances from polyurethane coating. *Environmental Science & Technology,* 51**,** 9979-9988.

LUTI, K. J. K. 2018. Mixture design of experiments for the optimization of carbon source for promoting undecylprodigiosin and actinorhodin production. *J. Pure Appl. Microbiol,* 12**,** 1783-1794.

LUTI, K. J. K. & YONIS, R. 2014. An induction of undecylprodigiosin production from *Streptomyces coelicolor* by elicitation with microbial cells using solid state fermentation. *Iraqi Journal of Science*, 1553-1562.

MAGNIN, A., ENTZMANN, L., POLLET, E. & AVÉROUS, L. 2021. Breakthrough in polyurethane bio-recycling: An efficient laccase-mediated system for the degradation of different types of polyurethanes. *Waste Management,* 132**,** 23-30.

MAGNIN, A., HOORNAERT, L., POLLET, E., LAURICHESSE, S., PHALIP, V. & AVÉROUS, L. 2019a. Isolation and characterization of different promising fungi for biological waste management of polyurethanes. *Microbial Biotechnology,* 12**,** 544-555.

MAGNIN, A., POLLET, E., PERRIN, R., ULLMANN, C., PERSILLON, C., PHALIP, V. & AVÉROUS, L. 2019b. Enzymatic recycling of thermoplastic polyurethanes: Synergistic effect of an esterase and an amidase and recovery of building blocks. *Waste Management,* 85**,** 141-150.

MAGNIN, A., POLLET, E., PHALIP, V. & AVÉROUS, L. 2020. Evaluation of biological degradation of polyurethanes. *Biotechnology advances,* 39**,** 107457.

MANIKANDAN, N. A., PAKSHIRAJAN, K. & PUGAZHENTHI, G. 2021. Techno-economic assessment of a sustainable and cost-effective bioprocess for large scale production of polyhydroxybutyrate. *Chemosphere,* 284**,** 131371.

MATSUMIYA, Y., MURATA, N., TANABE, E., KUBOTA, K. & KUBO, M. 2010. Isolation and characterization of an ether-type polyurethane-degrading micro-organism and analysis of degradation mechanism by Alternaria sp. *Journal of applied microbiology,* 108**,** 1946-1953.

MCADAM, B., BRENNAN FOURNET, M., MCDONALD, P. & MOJICEVIC, M. 2020. Production of polyhydroxybutyrate (PHB) and factors impacting its chemical and mechanical characteristics. *Polymers,* 12**,** 2908.

MOLITOR, R., BOLLINGER, A., KUBICKI, S., LOESCHCKE, A., JAEGER, K. E. & THIES, S. 2020. Agar plate-based screening methods for the identification of polyester hydrolysis by Pseudomonas species. *Microbial biotechnology,* 13**,** 274-284.

MOONEY, A., WARD, P. G. & O'CONNOR, K. E. 2006. Microbial degradation of styrene: biochemistry, molecular genetics, and perspectives for biotechnological applications. *Applied microbiology and biotechnology,* 72**,** 1-10.

MORADO, E. G., PATERSON, M. L., IVANOFF, D. G., WANG, H.-C., JOHNSON, A., DANIELS, D., RIZVI, A., SOTTOS, N. R. & ZIMMERMAN, S. C. 2023. End-of-life upcycling of polyurethanes using a room temperature, mechanism-based degradation. *Nature Chemistry,* 15**,** 569-577.

MORI, T., SAKIMOTO, M., KAGI, T. & SAKAI, T. 1996. Isolation and characterization of a strain of Bacillus megaterium that degrades poly (vinyl alcohol). *Bioscience, biotechnology, and biochemistry,* 60**,** 330-332.

MRIGWANI, A., THAKUR, B. & GUPTASARMA, P. 2022. Conversion of polyethylene terephthalate into pure terephthalic acid through synergy between a solid-degrading cutinase and a reaction intermediate-hydrolysing carboxylesterase. *Green Chemistry,* 24**,** 6707-6719.

NAKAJIMA-KAMBE, T., ONUMA, F., AKUTSU, Y. & NAKAHARA, T. 1997. Determination of the polyester polyurethane breakdown products and distribution of the polyurethane degrading enzyme of *Comamonas acidovorans* strain TB-35. *Journal of Fermentation and Bioengineering,* 83**,** 456-460.

NAKAJIMA-KAMBE, T., ONUMA, F., KIMPARA, N. & NAKAHARA, T. 1995. Isolation and characterization of a bacterium which utilizes polyester polyurethane as a sole carbon and nitrogen source. *FEMS Microbiology Letters,* 129**,** 39-42.

NAKAMIYA, K., SAKASITA, G., OOI, T. & KINOSHITA, S. 1997. Enzymatic degradation of polystyrene by hydroquinone peroxidase of *Azotobacter beijerinckii* HM121. *Journal of fermentation and bioengineering,* 84**,** 480-482.

NANDA, S., PATRA, B. R., PATEL, R., BAKOS, J. & DALAI, A. K. 2022. Innovations in applications and prospects of bioplastics and biopolymers: A review. *Environmental Chemistry Letters,* 20**,** 379-395.

NANDAKUMAR, A., CHUAH, J.-A. & SUDESH, K. 2021. Bioplastics: a boon or bane? *Renewable and Sustainable Energy Reviews,* 147**,** 111237.

NARANCIC, T., VERSTICHEL, S., REDDY CHAGANTI, S., MORALES-GAMEZ, L., KENNY, S. T., DE WILDE, B., BABU PADAMATI, R. & O'CONNOR, K. E. 2018. Biodegradable plastic blends create new possibilities for end-of-life management of plastics but they are not a panacea for plastic pollution. *Environmental science & technology,* 52**,** 10441-10452.

NIKODINOVIC-RUNIC, J., CASEY, E., DUANE, G. F., MITIC, D., HUME, A. R., KENNY, S. T. & O'CONNOR, K. E. 2011. Process analysis of the conversion of styrene to biomass and medium chain length polyhydroxyalkanoate in a two-phase bioreactor. *Biotechnology and bioengineering,* 108**,** 2447-2455.

NIKOLAIVITS, E., PANTELIC, B., AZEEM, M., TAXEIDIS, G., BABU, R., TOPAKAS, E., BRENNAN FOURNET, M. & NIKODINOVIC-RUNIC, J. 2021. Progressing plastics circularity: A review of mechano-biocatalytic approaches for waste plastic (re) valorization. *Frontiers in Bioengineering and Biotechnology,* 9**,** 535.

NIKOLAIVITS, E., TAXEIDIS, G., GKOUNTELA, C., VOUYIOUKA, S., MASLAK, V., NIKODINOVIC-RUNIC, J. & TOPAKAS, E. 2022. A polyesterase from the Antarctic bacterium *Moraxella* sp. degrades highly crystalline synthetic polymers. *Journal of Hazardous Materials,* 434**,** 128900.

NOMURA, N., SHIGENO-AKUTSU, Y., NAKAJIMA-KAMBE, T. & NAKAHARA, T. 1998. Cloning and sequence analysis of a polyurethane esterase of *Comamonas acidovorans* TB-35. *Journal of fermentation and bioengineering,* 86**,** 339-345.

NORTH, E. J. & HALDEN, R. U. 2013. Plastics and environmental health: the road ahead. *Reviews on environmental health,* 28**,** 1-8.

OU, D., NI, Y., LI, W., HE, W., WANG, L., HUANG, H. & PAN, Z. 2024. Psychrobacter species enrichment as potential microplastic degrader and the putative biodegradation mechanism in Shenzhen Bay sediment, China. *Journal of Hazardous Materials,* 464**,** 132971.

PARDO, I., JHA, R. K., BERMEL, R. E., BRATTI, F., GADDIS, M., MCINTYRE, E., MICHENER, W., NEIDLE, E. L., DALE, T. & BECKHAM, G. T. 2020. Gene amplification, laboratory evolution, and biosensor screening reveal MucK as a terephthalic acid transporter in *Acinetobacter baylyi* ADP1. *Metabolic Engineering,* 62**,** 260-274.

PARK, W. J., HWANGBO, M. & CHU, K.-H. 2023. Plastisphere and microorganisms involved in polyurethane biodegradation. *Science of The Total Environment,* 886**,** 163932.

PARKE, D., GARCIA, M. & ORNSTON, L. 2001. Cloning and genetic characterization of dca genes required for β-oxidation of straight-chain dicarboxylic acids in *Acinetobacter* sp. strain ADP1. *Applied and environmental microbiology,* 67**,** 4817-4827.

PLASTICSEUROPE. 2022. *Plastics - the Facts 2022*. https://plasticseurope.org/ [Pristupljeno 25.01.2024.].

PRANAMUDA, H. & TOKIWA, Y. 1999. Degradation of poly (L-lactide) by strains belonging to genus Amycolatopsis. *Biotechnology Letters,* 21**,** 901-905.

PROVENCHER, J. F., BOND, A. L., AVERY-GOMM, S., BORRELLE, S. B., REBOLLEDO, E. L. B., HAMMER, S., KÜHN, S., LAVERS, J. L., MALLORY, M. L. & TREVAIL, A. 2017. Quantifying ingested debris in marine megafauna: a review and recommendations for standardization. *Analytical Methods,* 9**,** 1454-1469.

PUIGGENÉ, Ò., ESPINOSA, M. J. C., SCHLOSSER, D., THIES, S., JEHMLICH, N., KAPPELMEYER, U., SCHREIBER, S., WIBBERG, D., KALINOWSKI, J. & HARMS, H. 2022. Extracellular degradation of a polyurethane oligomer involving outer membrane vesicles and further insights on the degradation of 2, 4-diaminotoluene in *Pseudomonas capeferrum* TDA1. *Scientific Reports,* 12**,** 2666.

QI, X., MA, Y., CHANG, H., LI, B., DING, M. & YUAN, Y. 2021. Evaluation of PET degradation using artificial microbial consortia. *Frontiers in microbiology,* 12**,** 778828.

QIU, L., YIN, X., LIU, T., ZHANG, H., CHEN, G. & WU, S. 2020. Biodegradation of bis (2-hydroxyethyl) terephthalate by a newly isolated *Enterobacter* sp. HY1 and characterization of its esterase properties. *Journal of basic microbiology,* 60**,** 699-711.

RABOT, C., CHEN, Y., BIJLANI, S., CHIANG, Y. M., OAKLEY, C. E., OAKLEY, B. R., WILLIAMS, T. J. & WANG, C. C. 2023a. Conversion of Polyethylenes into Fungal Secondary Metabolites. *Angewandte Chemie International Edition,* 62**,** e202214609.

RABOT, C., CHEN, Y., LIN, S.-Y., MILLER, B., CHIANG, Y.-M., OAKLEY, C. E., OAKLEY, B. R., WANG, C. C. & WILLIAMS, T. J. 2023b. Polystyrene Upcycling into Fungal Natural Products and a Biocontrol Agent. *Journal of the American Chemical Society,* 145**,** 5222-5230.

RAGAERT, K., DELVA, L. & VAN GEEM, K. 2017. Mechanical and chemical recycling of solid plastic waste. *Waste management,* 69**,** 24-58.

RIBITSCH, D., HEUMANN, S., TROTSCHA, E., HERRERO ACERO, E., GREIMEL, K., LEBER, R., BIRNER-GRUENBERGER, R., DELLER, S., EITELJOERG, I. & REMLER, P. 2011. Hydrolysis of polyethyleneterephthalate by p-nitrobenzylesterase from *Bacillus subtilis*. *Biotechnology progress,* 27**,** 951-960.

ROBERTS, C., EDWARDS, S., VAGUE, M., LEÓN-ZAYAS, R., SCHEFFER, H., CHAN, G., SWARTZ, N. A. & MELLIES, J. L. 2020. Environmental consortium containing *Pseudomonas* and *Bacillus* species synergistically degrades polyethylene terephthalate plastic. *Msphere,* 5**,** e01151-20.

ROMMI, K., RAHIKAINEN, J., VARTIAINEN, J., HOLOPAINEN, U., LAHTINEN, P., HONKAPÄÄ, K. & LANTTO, R. 2016. Potato peeling costreams as raw materials for biopolymer film preparation. *Journal of Applied Polymer Science,* 133.

RONG, Z., DING, Z.-H., WU, Y.-H. & XU, X.-W. 2024. Degradation of low-density polyethylene by the bacterium *Rhodococcus* sp. C-2 isolated from seawater. *Science of The Total Environment,* 907**,** 167993.

RONKVIST, Å. M., XIE, W., LU, W. & GROSS, R. A. 2009. Cutinase-catalyzed hydrolysis of poly (ethylene terephthalate). *Macromolecules,* 42**,** 5128-5138.

RÜTHI, J., CERRI, M., BRUNNER, I., STIERLI, B., SANDER, M. & FREY, B. 2023. Discovery of plastic-degrading microbial strains isolated from the alpine and Arctic terrestrial plastisphere. *Frontiers in Microbiology,* 14**,** 1178474.

SANLUIS-VERDES, A., COLOMER-VIDAL, P., RODRIGUEZ-VENTURA, F., BELLO-VILLARINO, M., SPINOLA-AMILIBIA, M., RUIZ-LOPEZ, E., ILLANES-VICIOSO, R., CASTROVIEJO, P., AIESE CIGLIANO, R. & MONTOYA, M. 2022. Wax worm saliva and the enzymes therein are the key to polyethylene degradation by *Galleria mellonella*. *Nature Communications,* 13**,** 1-11.

SANTO, M., WEITSMAN, R. & SIVAN, A. 2013. The role of the copper-binding enzyme–laccase–in the biodegradation of polyethylene by the actinomycete *Rhodococcus ruber*. *International Biodeterioration & Biodegradation,* 84**,** 204-210.

SCHMIDT, J., WEI, R., OESER, T., DEDAVID E SILVA, L. A., BREITE, D., SCHULZE, A. & ZIMMERMANN, W. 2017. Degradation of polyester polyurethane by bacterial polyester hydrolases. *Polymers,* 9**,** 65.

SHAH, Z., GULZAR, M., HASAN, F. & SHAH, A. A. 2016. Degradation of polyester polyurethane by an indigenously developed consortium of *Pseudomonas* and *Bacillus* species isolated from soil. *Polymer Degradation and Stability,* 134**,** 349-356.

SHAH, Z., HASAN, F., KRUMHOLZ, L., AKTAS, D. F. & SHAH, A. A. 2013a. Degradation of polyester polyurethane by newly isolated *Pseudomonas aeruginosa* strain MZA-85 and analysis of degradation products by GC–MS. *International Biodeterioration & Biodegradation,* 77**,** 114-122.

SHAH, Z., KRUMHOLZ, L., AKTAS, D. F., HASAN, F., KHATTAK, M. & SHAH, A. A. 2013b. Degradation of polyester polyurethane by a newly isolated soil bacterium, *Bacillus subtilis* strain MZA-75. *Biodegradation,* 24**,** 865-877.

SHI, J., ZENG, Y. J., ZHANG, B., SHAO, F. L., CHEN, Y. C., XU, X., SUN, Y., XU, Q., TAN, R. X. & GE, H. M. 2019. Comparative genome mining and heterologous expression of an orphan NRPS gene cluster direct the production of ashimides. *Chemical Science,* 10**,** 3042-3048.

SHIRKE, A. N., WHITE, C., ENGLAENDER, J. A., ZWARYCZ, A., BUTTERFOSS, G. L., LINHARDT, R. J. & GROSS, R. A. 2018. Stabilizing leaf and branch compost cutinase (LCC) with glycosylation: Mechanism and effect on PET hydrolysis. *Biochemistry,* 57**,** 1190-1200.

SINGHVI, N., SINGH, P., PRAKASH, O., GUPTA, V., LAL, S., BECHTHOLD, A., SINGH, Y., SINGH, R. K. & LAL, R. 2021. Differential mass spectrometry-based proteome analyses unveil major regulatory hubs in rifamycin B production in *Amycolatopsis mediterranei. Journal of Proteomics,* 239**,** 104168.

SKARIYACHAN, S., PATIL, A. A., SHANKAR, A., MANJUNATH, M., BACHAPPANAVAR, N. & KIRAN, S. 2018. Enhanced polymer degradation of polyethylene and polypropylene by novel thermophilic consortia of *Brevibacillus* sps. and *Aneurinibacillus* sp. screened from waste management landfills and sewage treatment plants. *Polymer Degradation and Stability,* 149**,** 52-68.

SON, H. F., CHO, I. J., JOO, S., SEO, H., SAGONG, H.-Y., CHOI, S. Y., LEE, S. Y. & KIM, K.-J. 2019. Rational protein engineering of thermo-stable PETase from Ideonella sakaiensis for highly efficient PET degradation. *ACS Catalysis,* 9**,** 3519-3526.

SONNENDECKER, C., OESER, J., RICHTER, P. K., HILLE, P., ZHAO, Z., FISCHER, C., LIPPOLD, H., BLÁZQUEZ-SÁNCHEZ, P., ENGELBERGER, F. & RAMÍREZ-SARMIENTO, C. A. 2022. Low carbon footprint recycling of post-consumer PET plastic with a metagenomic polyester hydrolase. *ChemSusChem,* 15**,** e202101062.

SOUD, S. A. 2019. Biodegradation of Polyethylene LDPE plastic waste using Locally Isolated *Streptomyces* sp. *Journal of Pharmaceutical Sciences and Research,* 11**,** 1333-1339.

SOUZA, C. C. D., GUIMARÃES, J. M., PEREIRA, S. D. S. & MARIÚBA, L. A. M. 2021. The multifunctionality of expression systems in *Bacillus subtilis*: Emerging devices for the production of recombinant proteins. *Experimental Biology and Medicine,* 246**,** 2443-2453.

SPASIC, J., MANDIC, M., DJOKIC, L. & NIKODINOVIC-RUNIC, J. 2018. *Streptomyces* spp. in the biocatalysis toolbox. *Applied microbiology and biotechnology,* 102**,** 3513-3536.

STANKOVIC, N., RADULOVIC, V., PETKOVIC, M., VUCKOVIC, I., JADRANIN, M., VASILJEVIC, B. & NIKODINOVIC-RUNIC, J. 2012. *Streptomyces* sp. JS520 produces exceptionally high quantities of undecylprodigiosin with antibacterial, antioxidative, and UV-protective properties. *Applied microbiology and biotechnology,* 96**,** 1217-1231.

STEGMANN, P., DAIOGLOU, V., LONDO, M., VAN VUUREN, D. P. & JUNGINGER, M. 2022. Plastic futures and their CO2 emissions. *Nature,* 612**,** 272-276.

SUH, D., PARK, O. & YOON, K. 2000. The properties of unsaturated polyester based on the glycolyzed poly (ethylene terephthalate) with various glycol compositions. *Polymer,* 41**,** 461-466.

SULAIMAN, S., YAMATO, S., KANAYA, E., KIM, J.-J., KOGA, Y., TAKANO, K. & KANAYA, S. 2012. Isolation of a novel cutinase homolog with polyethylene terephthalate-degrading activity from leaf-branch compost by using a metagenomic approach. *Appl. Environ. Microbiol.,* 78**,** 1556-1562.

SUMMERS, R. G., ALI, A., SHEN, B., WESSEL, W. A. & HUTCHINSON, C. R. 1995. Malonyl-coenzyme A: acyl carrier protein acyltransferase of *Streptomyces glaucescens*: a possible link between fatty acid and polyketide biosynthesis. *Biochemistry,* 34**,** 9389-9402.

TAN, Y., HENEHAN, G. T., KINSELLA, G. K. & RYAN, B. J. 2021. An extracellular lipase from *Amycolatopsis mediterannei* is a cutinase with plastic degrading activity. *Computational and structural biotechnology journal,* 19**,** 869-879.

TANAKA, K., TAKADA, H., YAMASHITA, R., MIZUKAWA, K., FUKUWAKA, M.-A. & WATANUKI, Y. 2013. Accumulation of plastic-derived chemicals in tissues of seabirds ingesting marine plastics. *Marine pollution bulletin,* 69**,** 219-222.

TAXEIDIS, G., NIKOLAIVITS, E., SIAPERAS, R., GKOUNTELA, C., VOUYIOUKA, S., PANTELIC, B., NIKODINOVIC-RUNIC, J. & TOPAKAS, E. 2023. Triggering and identifying the polyurethane and polyethylene-degrading machinery of filamentous fungi secretomes. *Environmental Pollution,* 325**,** 121460.

THAKUR, S., CHAUDHARY, J., SHARMA, B., VERMA, A., TAMULEVICIUS, S. & THAKUR, V. K. 2018. Sustainability of bioplastics: Opportunities and challenges. *Current opinion in Green and Sustainable chemistry,* 13**,** 68-75.

THEN, J., WEI, R., OESER, T., BARTH, M., BELISÁRIO-FERRARI, M. R., SCHMIDT, J. & ZIMMERMANN, W. 2015. Ca2+ and Mg2+ binding site engineering increases the degradation of polyethylene terephthalate films by polyester hydrolases from Thermobifida fusca. *Biotechnology Journal,* 10**,** 592-598.

THIOUNN, T. & SMITH, R. C. 2020. Advances and approaches for chemical recycling of plastic waste. *Journal of Polymer Science,* 58**,** 1347-1364.

THIRUNAVUKARASU, K., PURUSHOTHAMAN, S., GOWTHAMAN, M., NAKAJIMA-KAMBE, T., ROSE, C. & KAMINI, N. 2015. Utilization of fish meal and fish oil for production of *Cryptococcus* sp. MTCC 5455 lipase and hydrolysis of polyurethane thereof. *Journal of food science and technology,* 52**,** 5772-5780.

THOMSEN, T. B., HUNT, C. J. & MEYER, A. S. 2022. Influence of substrate crystallinity and glass transition temperature on enzymatic degradation of polyethylene terephthalate (PET). *New Biotechnology,* 69**,** 28-35.

TIAN, H., YAN, J., RAJULU, A. V., XIANG, A. & LUO, X. 2017. Fabrication and properties of polyvinyl alcohol/starch blend films: Effect of composition and humidity. *International Journal of Biological Macromolecules,* 96**,** 518-523.

TISO, T., NARANCIC, T., WEI, R., POLLET, E., BEAGAN, N., SCHRÖDER, K., HONAK, A., JIANG, M., KENNY, S. T. & WIERCKX, N. 2021. Towards bio-upcycling of polyethylene terephthalate. *Metabolic engineering,* 66**,** 167-178.

TORRES, F., TRONCOSO, O., TORRES, C., DÍAZ, D. & AMAYA, E. 2011. Biodegradability and mechanical properties of starch films from Andean crops. *International journal of biological macromolecules,* 48**,** 603-606.

TOURNIER, V., TOPHAM, C., GILLES, A., DAVID, B., FOLGOAS, C., MOYA-LECLAIR, E., KAMIONKA, E., DESROUSSEAUX, M.-L., TEXIER, H. & GAVALDA, S. 2020. An engineered PET depolymerase to break down and recycle plastic bottles. *Nature,* 580**,** 216-219.

UEKERT, T., DESVEAUX, J. S., SINGH, A., NICHOLSON, S. R., LAMERS, P., GHOSH, T., MCGEEHAN, J. E., CARPENTER, A. C. & BECKHAM, G. T. 2022. Life cycle assessment of enzymatic poly (ethylene terephthalate) recycling. *Green Chemistry,* 24**,** 6531-6543.

URBANEK, A. K., RYMOWICZ, W. & MIROŃCZUK, A. M. 2018. Degradation of plastics and plastic-degrading bacteria in cold marine habitats. *Applied microbiology and biotechnology,* 102**,** 7669-7678.

UTOMO, R. N. C., LI, W.-J., TISO, T., EBERLEIN, C., DOEKER, M., HEIPIEPER, H. J., JUPKE, A., WIERCKX, N. & BLANK, L. M. 2020. Defined microbial mixed culture for utilization of polyurethane monomers. *ACS Sustainable Chemistry & Engineering,* 8**,** 17466-17474.

VARGAS-SUÁREZ, M., SAVÍN-GÁMEZ, A., DOMÍNGUEZ-MALFAVÓN, L., SÁNCHEZ-REYES, A., QUIRASCO-BARUCH, M. & LOZA-TAVERA, H. 2021. Exploring the polyurethanolytic activity and microbial composition of landfill microbial communities. *Applied Microbiology and Biotechnology,* 105**,** 7969-7980.

VERT, M., DOI, Y., HELLWICH, K.-H., HESS, M., HODGE, P., KUBISA, P., RINAUDO, M. & SCHUÉ, F. 2012. Terminology for biorelated polymers and applications (IUPAC Recommendations 2012). *Pure and Applied Chemistry,* 84**,** 377-410.

VOGT, B. D., STOKES, K. K. & KUMAR, S. K. 2021. Why is recycling of postconsumer plastics so challenging? *ACS Applied Polymer Materials,* 3**,** 4325-4346.

VOJNOVIC, S., ALEKSIC, I., ILIC-TOMIC, T., STEVANOVIC, M. & NIKODINOVIC-RUNIC, J. 2024. *Bacillus* and *Streptomyces* spp. as hosts for production of industrially relevant enzymes. *Applied Microbiology and Biotechnology,* 108**,** 185.

VOLODINA, E., RABERG, M. & STEINBÜCHEL, A. 2016. Engineering the heterotrophic carbon sources utilization range of *Ralstonia eutropha* H16 for applications in biotechnology. *Critical reviews in biotechnology,* 36**,** 978-991.

WAGENER, S., VÖLKER, T., DE SPIRT, S., ERNST, H. & STAHL, W. 2012. 3, 3'-Dihydroxyisorenieratene and isorenieratene prevent UV-induced DNA damage in human skin fibroblasts. *Free Radical Biology and Medicine,* 53**,** 457-463.

WALLBANK, J. A., LEAR, G., KINGSBURY, J. M., WEAVER, L., DOAKE, F., SMITH, D. A., AUDRÉZET, F., MADAY, S. D., GAMBARINI, V. & DONALDSON, L. 2022. Into the Plastisphere, where only the generalists thrive: early insights in plastisphere microbial community succession. *Frontiers in Marine Science,* 9**,** 841142.

WANG, D., LIN, J., LIN, J., WANG, W. & LI, S. 2019. Biodegradation of petroleum hydrocarbons by *Bacillus subtilis* BL-27, a strain with weak hydrophobicity. *Molecules,* 24**,** 3021.

WANG, N., GUAN, F., LV, X., HAN, D., ZHANG, Y., WU, N., XIA, X. & TIAN, J. 2020. Enhancing secretion of polyethylene terephthalate hydrolase PETase in *Bacillus subtilis* WB600 mediated by the SPamy signal peptide. *Letters in Applied Microbiology,* 71**,** 235-241.

WEI, R., BREITE, D., SONG, C., GRÄSING, D., PLOSS, T., HILLE, P., SCHWERDTFEGER, R., MATYSIK, J., SCHULZE, A. & ZIMMERMANN, W. 2019. Biocatalytic degradation efficiency of postconsumer polyethylene terephthalate packaging determined by their polymer microstructures. *Advanced Science,* 6**,** 1900491.

WEI, R. & ZIMMERMANN, W. 2017. Microbial enzymes for the recycling of recalcitrant petroleum-based plastics: how far are we? *Microbial biotechnology,* 10**,** 1308-1322.

WHICHER, J. R., FLOROVA, G., SYDOR, P. K., SINGH, R., ALHAMADSHEH, M., CHALLIS, G. L., REYNOLDS, K. A. & SMITH, J. L. 2011. Structure and function of the RedJ protein, a thioesterase from the prodiginine biosynthetic pathway in *Streptomyces coelicolor*. *Journal of Biological Chemistry,* 286**,** 22558-22569.

XI, X., NI, K., HAO, H., SHANG, Y., ZHAO, B. & QIAN, Z. 2021. Secretory expression in *Bacillus subtilis* and biochemical characterization of a highly thermostable polyethylene terephthalate hydrolase from bacterium HR29. *Enzyme and microbial technology,* 143**,** 109715.

YANG, X.-G., WEN, P.-P., YANG, Y.-F., JIA, P.-P., LI, W.-G. & PEI, D.-S. 2023. Plastic biodegradation by in vitro environmental microorganisms and in vivo gut microorganisms of insects. *Frontiers in Microbiology,* 13**,** 1001750.

YANG, Y., MALTEN, M., GROTE, A., JAHN, D. & DECKWER, W. D. 2007. Codon optimized *Thermobifida fusca* hydrolase secreted by *Bacillus megaterium*. *Biotechnology and bioengineering,* 96**,** 780-794.

YASTREBOVA, O., MALYSHEVA, A. & PLOTNIKOVA, E. 2022. Halotolerant Terephthalic Acid-Degrading Bacteria of the Genus *Glutamicibacter*. *Applied Biochemistry and Microbiology,* 58**,** 590-597.

YOSHIDA, S., HIRAGA, K., TAKEHANA, T., TANIGUCHI, I., YAMAJI, H., MAEDA, Y., TOYOHARA, K., MIYAMOTO, K., KIMURA, Y. & ODA, K. 2016. A bacterium that degrades and assimilates poly (ethylene terephthalate). *Science,* 351**,** 1196-1199.

YU, K. H., ZHANG, Y., LI, D., MONTENEGRO-MARIN, C. E. & KUMAR, P. M. 2021. Environmental planning based on reduce, reuse, recycle and recover using artificial intelligence. *Environmental Impact Assessment Review,* 86**,** 106492.

ZABALA, D., BRAÑA, A. F., SALAS, J. A. & MÉNDEZ, C. 2016. Increasing antibiotic production yields by favoring the biosynthesis of precursor metabolites glucose-1-phosphate and/or malonyl-CoA in *Streptomyces* producer strains. *The Journal of Antibiotics,* 69**,** 179-182.

ZALASIEWICZ, J., WATERS, C. N., DO SUL, J. A. I., CORCORAN, P. L., BARNOSKY, A. D., CEARRETA, A., EDGEWORTH, M., GAŁUSZKA, A., JEANDEL, C. & LEINFELDER, R. 2016. The geological cycle of plastics and their use as a stratigraphic indicator of the Anthropocene. *Anthropocene,* 13**,** 4-17.

ZAMPOLLI, J., MANGIAGALLI, M., VEZZINI, D., LASAGNI, M., AMI, D., NATALELLO, A., ARRIGONI, F., BERTINI, L., LOTTI, M. & DI GENNARO, P. 2023. Oxidative degradation of polyethylene by two novel laccase-like multicopper oxidases from *Rhodococcus opacus* R7. *Environmental Technology & Innovation,* 32**,** 103273.

ZHANG, H., DIERKES, R. F., PEREZ-GARCIA, P., COSTANZI, E., DITTRICH, J., CEA, P. A., GURSCHKE, M., APPLEGATE, V., PARTUS, K. & SCHMEISSER, C. 2023. The metagenome-derived esterase PET40 is highly promiscuous and hydrolyses polyethylene terephthalate (PET). *The FEBS Journal*.

ZHANG, K., HU, J., YANG, S., XU, W., WANG, Z., ZHUANG, P., GROSSART, H.-P. & LUO, Z. 2022. Biodegradation of Polyester Polyurethane by the Marine Fungus *Cladosporium halotolerans* 6UPA1. *Journal of Hazardous Materials***,** 129406.

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Brana P. Pantelić je rođen 22.12.1995. godine u Beogradu. Osnovnu i XIII beogradsku gimnaziju završio je sa odličnim uspehom. Biološki fakultet Univerziteta u Beogradu, studijska program Molekularna biologija i fiziologija upisao je 2014. godine, a diplomirao je 2018. godine sa prosečnom ocenom 9.35. Iste godine nastavlja master akademske studije na studijskom programu Molekularna biologija i fiziologija na Biološkom fakultetu, modul Biotehnologija i genetičko inženjerstvo koji završava sa prosečnom ocenom 10 i odbranom master rada " Karakterizacija kutinaze iz soja *Streptomyces* sp. BV286". Nakon završenih master studija, 2019. godine upisuje doktorske studije na Biološkom fakultetu, studijski program Molekularna biologija, modul Molekularna biologija prokariota. Od januara 2020. godine Brana Pantelić je zaposlen na Institutu za molekularnu genetiku i genetičko inženjerstvo, Univerzitet u Beogradu u Grupi za eko-biotehnologiju i razvoj lekova kao istraživač pripravnik, a od jula 2022. godine je u zvanju istraživač saradnik. Brana Pantelić učestvovao je u realizaciji H2020 projekta BioICEP (eng. *Bio Innovation of a Circular Economy for Plastics*, grant agreement No. 870292) i BioECOLogics projekta (eng. *Value-added biologics through eco-sustainable routes*, grant agreement No. 7730810) finansiranog od strane Fonda za Nauku Republike Srbije kao i niza manjih istraživačkih, inovativnih i projekata za popularizaciju nauke.

Brana Pantelić je član Udruženja mikrobiologa Srbije, Srpskog društva za molekularnu biologiju, Evropske federacije za biotehnologiju (EFB) i Evropskog društva za primenjenu biokatalizu (ESAB). Kao autor ili koautor objavio je 8 naučnih radova u vodećim međunarodnim časopisima kategorija M21a (2), M21 (4) i M22 (2) i 14 saopštenja na naučnim skupovima.

Naučni radovi iz oblasti doktorske disertacije:

1. Araujo, J. A., Taxeidis, G., Pereira, E. H., Azeem, M., **Pantelic, B.**, Jeremic, S., Ponjavic, M., Chen, Y., Mojicevic, M., Nikodinovic- Runic, J., Topakas, E., Brennan Fournet, M., 2024. Biotechnological model for ubiquitous mixed petroleum-and bio-based plastics degradation and upcycling into bacterial nanocellulose. *Journal of Cleaner Production*, 141025.

2. Herrera, D.A.G., Mojicevic, M., **Pantelic, B.**, Joshi, A., Collins, C., Batista, M., Torres, C., Freitas, F., Murray, P., Nikodinovic-Runic, J., 2023. Exploring Microorganisms from Plastic-Polluted Sites: Unveiling Plastic Degradation and PHA Production Potential, *Microorganisms*, 11 2914.

3. Nikolaivits, E., **Pantelic, B.**, Azeem, M., Taxeidis, G., Babu, R., Topakas, E., Brennan Fournet, M., Nikodinovic-Runic, J., 2021. Progressing plastics circularity: A review of mechano-biocatalytic approaches for waste plastic (re) valorization, *Frontiers in Bioengineering and Biotechnology*, 9 535.

4. **Pantelic, B.**, Ponjavic, M., Jankovic, V., Aleksic, I., Stevanovic, S., Murray, J., Fournet, M.B., Nikodinovic-Runic, J., 2021. Upcycling biodegradable PVA/starch film to a bacterial biopigment and biopolymer, *Polymers*, 13 3692.

5. **Pantelic, B.**, Skaro Bogojevic, S., Milivojevic, D., Ilic-Tomic, T., Lončarević, B., Beskoski, V., Maslak, V., Guzik, M., Makryniotis, K., Taxeidis, G., 2023. Set of Small Molecule Polyurethane (PU) Model Substrates: Ecotoxicity Evaluation and Identification of PU Degrading Biocatalysts, *Catalysts*, 13 278.

6. **Pantelic, B.**, Araujo, J.A., Jeremic, S., Azeem, M., Attallah, O.A., Siaperas, R., Mojicevic, M., Chen, Y., Fournet, M.B., Topakas, E., 2023. A novel *Bacillus subtilis* BPM12 with high bis (2 hydroxyethyl) terephthalate hydrolytic activity efficiently interacts with virgin and mechanically recycled polyethylene terephthalate, *Environmental Technology & Innovation*, 32 103316.

7. **Pantelic, B.**, Siaperas, R., Budin, C., de Boer, T., Topakas, E., Nikodinovic-Runic, J., 2024. Proteomic Examination of Polyester-Polyurethane Degradation by *Streptomyces* sp. PU10: Diverting Polyurethane Intermediates to Secondary Metabolite Production, *Microbial Biotechnology,* 00, e14445.

8. Taxeidis, G., Nikolaivits, E., Siaperas, R., Gkountela, C., Vouyiouka, S., **Pantelic, B.**, Nikodinovic-Runic, J., Topakas, E., 2023. Triggering and identifying the polyurethane and polyethylene-degrading machinery of filamentous fungi secretomes, *Environmental Pollution*, 325 121460.

Прилог 1.

Изјава о ауторству

Потписани-а **Брана П. Пантелић**

број индекса **М3004/2019**

Изјављујем

да је докторска дисертација под насловом

<u>Изолација бактерија и карактеризација њихових ензима са циљем развијања биокатализатора за биодеградацију пластике</u>

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Потпис аутора

У Београду, 12.03.2024.

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Прилог 2.

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора **Брана П. Пантелић**

Број индекса **M3004/2019**

Студијски програм **Молекуларна Биологија**

Наслов рада **Изолација бактерија и карактеризација њихових ензима са циљем развијања биокатализатора за биодеградацију пластике**

Ментор **др Јасмина Никодиновић-Рунић**, др Бранко Јовчић

Потписани/а **Брана П. Пантелић**

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду**.

Дозвољавам да се објаве моји лични подаци везани за добијање академског звања доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис аутора

У Београду, 12.03.2024.

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Прилог 3.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић" да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

Изолација бактерија и карактеризација њихових ензима са циљем развијања биокатализатора за биодеградацију пластике

која је моје ауторско дело.

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