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Badanie zdolności fizjologicznych drożdży *Yarrowia lipolytica* do rozkładu tworzyw sztucznych

Investigation of the ability of the *Yarrowia lipolytica* yeast to degrade plastic materials

Praca Doktorska wykonana pod kierunkiem:

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Publikacja 2 (P2):

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Publikacja 3 (P3):

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IF= 10,753; MEiN= 200

Całkowity współczynnik IF publikacji, które wchodzą w skład pracy doktorskiej wynosi 32,477 oraz 640 punktów ministerialnych. Wartości współczynnika IF podane zostały w zgodzie z rokiem opublikowania. Punkty MEiN dla wszystkich artykułów zostały podane zgodnie z rokiem publikacji komunikatu Ministra Edukacji i Nauki z dnia 01.12.2021 r.

Streszczenie

Zanieczyszczenie planety odpadami z tworzyw sztucznych jest w ostatnich latach coraz bardziej dostrzegalne. Zwiększona produkcja i wykorzystanie tych materiałów w wielu dziedzinach przemysłu, połączone z niedopracowanym systemem zarządzania odpadami sprawiły, że coraz więcej ekosystemów odczuwa bolesne skutki akumulacji tychże śmieci. Jednym z najbardziej powszechnych tworzyw sztucznych na świecie jest poli(tereftalan etylenu) (PET), polimer o bardzo dobrych właściwościach fizyko-chemicznych, który jest uważany za niebiodegradowalny. Obecnie, naukowcy prowadzą wzmożone badania mające na celu rozwiązać ten realny i narastający problem a dotychczasowym rezultatem było określenie enzymów z klasy hydrolaz, takich jak kutynazy, lipazy i PETaza, jako zdolnych do hydrolizy wiązań estrowych obecnych z poliestrach (P1). Niniejsza praca skupia się na badaniu zdolności zmodyfikowanych drożdży *Yarrowia lipolytica* do rozkładu tworzyw sztucznych i ma na celu wprowadzenie nowatorskiej metody degradacji poliestrów bezpośrednio w hodowli mikroorganizmów.

W pierwszej fazie badań koncentrowano się na degradacji poliestrów alifatycznych, w której wykorzystano szczep drożdży *Y. lipolytica* produkujący pozakomórkowo kutynazy z *F. solani* i *T. reesei* z koekspresją natywnej lipazy (P2). Badania w tym zakresie dotyczyły aktywności enzymatycznej enzymów obecnych w supernatancie pochodzącym z hodowli, ich zdolności do tworzenia stref przejaśnień na emulgowanym podłożu poliestrowym (poli ε-kaprolaktonu; PCL), ilościowego oznaczania ilości uwalnianego ε-kaprolaktonu w procesie rozkładu oraz ubytku masy biodegradowalnych folii z tworzywa sztucznego po hodowli ze zmodyfikowanymi szczepami. W niniejszych badaniach ustalono, że szczepem wyróżniającym się wysokim poziomem biodegradacji poliestrów jest szczep *Y. lipolytica* z nadekspresją kutynazy z *F. solani* (AJD2 pAD CUT_FS), wykorzystany również do biodegradacji PET (P3). Ponieważ PETaza z *Ideonella sakaiensis* nie wykazuje zdolności do hydrolizy wiązań estrowych obecnych w poliestrach alifatycznych (takich jak PCL), szczep produkujący ten enzym, wraz ze szczepem wybranym w pierwszym etapie badań, został wykorzystany do oceny zdolności degradacyjnych tworzywa PET (P4).

Proces rozkładu PET został przeprowadzono bezpośrednio w hodowli zmodyfikowanych szczepów *Y. lipolytica*. Dodatkowo zbadano wpływ suplementacji na wydajność degradacji, stosując różne stężenia soli oraz oliwy z oliwek. Efektywność degradacji tworzyw sztucznych określono na podstawie ilości uwolnionych produktów

hydrolizy PET, takich jak kwas tereftalowy (TPA) oraz kwas mono-(2-hydroksyetylowy)tereftalowy (MHET) przy użyciu ultrasprawnej chromatografii cieczowej (UPLC). Dodatkowo zbadano również zdolność do asymilacji końcowych produktów degradacji PET (takich jak TPA, EG) przez *Y. lipolytica* i porównano zdolność do hydrolizy MHET przez zmodyfikowane szczepy kutynazę z *F. solani* i PETazę z *I. sakaiensis*. Ponadto przeprowadzono hodowlę drożdży z folią PET, a strukturę jej powierzchni na folii sprawdzono za pomocą skaningowego mikroskopu elektronowego (SEM).

Badania przeprowadzone w ramach tej pracy wskazują, że *Y. lipolytica* jest odpowiednim kandydatem, który może być wykorzystany jako organizm gospodarza do pozakomórkowej produkcji enzymów hydrolizujących poliestry. Ze względu na stabilizację pH podłoża hodowlanego po 72 h hodowli w zakresie optymalnych warunków środowiskowych dla obu stosowanych enzymów (pH 8,0-8,5) wykazano, że proces degradacji tworzyw sztucznych może być wydajnie przeprowadzony bezpośrednio w hodowli mikroorganizmów. Dodatkowo w naszych badaniach wykazaliśmy, że *Y. lipolytica* jest zdolna do asymilacji glikolu etylenowego (EG), który wraz z TPA jest końcowym produktem hydrolizy tego polimeru.

Streszczenie w języku angielskim (Abstract)

Pollution of the planet with plastic waste has become increasingly apparent in recent years. The increased production and use of such materials in a wide range of industries, combined with an underdeveloped waste management system, has resulted in an increasing number of ecosystems being harmed by the accumulation of this litter. One of the most common plastics in the world is poly(ethylene terephthalate) (PET), which is considered a non-biodegradable polymer with outstanding physical and chemical properties. Currently, scientists are undertaking intensive research aiming at solving this feasible and escalating problem and to date, the results were the identification of enzymes from the hydrolase class, such as cutinases, lipases and PETase, as capable of hydrolyzing the ester bonds present in polyesters (P1|). The current work focuses on examining the ability of modified *Yarrowia lipolytica* yeast to degrade plastics and aims to introduce a novel method for degrading polyesters directly in microbial culture.

The increased production and use of plastics in recent years has resulted in their significant accumulation in the environment, which negatively affects the entire ecosystem. One of the world's most common plastic material is poly(ethylene terephthalate) (PET) is one of the world's most widely used plastic material with wide range of usage i.e. packaging, construction and automotive industry. To the date, the enzymes from hydrolase class such as cutinases, lipases and PETase were classified as capable to hydrolyze ester bonds present in polyesters.

First, the research was focused on aliphatic plastic degradation, where *Y. lipolytica* yeast strain extracellularly producing cutinases from *F. solani* and *T. reesei* with co-expression with native *Y. lipolytica* lipase (P2). The work within this scope focused on investigation the enzymatic activity of the enzymes present in the culture's supernatant, the capacity to form clear zones on the emulsified polyester substrate medium, the quantitative assay of the amount of released ε-caprolactone during decomposition process, and the mass loss of biodegradable plastic films. In this study, we have determined the best candidate for further work with more challenging plastic material is a strain producing cutinase from *F. solani*, employed also for biodegradation of PET (P3). Considering that PETase from *Ideonella sakaiensis* does not exhibit the ability to hydrolyze ester bonds present in aliphatic polyesters, the strain producing this enzyme, along with the strain selected in the first stage of the studies, was used to examine degradation of PET material (P4). The investigation of the capacity

to hydrolyze this plastic were performed directly in the culture of the above-mentioned *Y. lipolytica* engineered strains. In addition, we have tested the supplementation influence on the degradation efficiency with the use of various salts and olive oil at different concentrations. Plastic degradation capacity by *Y. lipolytica* strains was determined based on the amount of released PET hydrolysis products such as terephthalic acid (TPA), mono-(2-hydroxyethyl) terephthalic acid (MHET) and with the use of ultraperformance liquid chromatography (UPLC). Furthermore, we have also investigated the ability to assimilate terminal PET degradation products by *Y. lipolytica* and compared the ability to hydrolyze MHET by modified strains producing cutinase from *F. solani* and PETase from *I. sakaiensis*. Finally, the yeast culture with PET film was carried out and the structure of the film was verified by scanning electron microscopy.

The research conducted in this thesis indicates that *Y. lipolytica* is a highly suitable candidate which may be used as a host organism for the extracellular production of enzymes hydrolyzing polyesters. Due to the stabilization of the pH of the culture medium after 72 h of culture within the range of optimal environmental conditions for both enzymes used (pH 8.0-8.5), plastic degradation process can be accomplished directly in the microbial culture. Additionally, in our study we have demonstrated that *Y. lipolytica* is able to assimilate ethylene glycol (EG), which, along with TPA, is the final product of the hydrolysis of this polymer.

1. Wstęp

Zwiększona produkcja i konsumpcja tworzyw sztucznych w ostatnich latach przyczyniła się do poważnego zanieczyszczenia środowiska powstającymi z nich odpadami. Od 1950 roku wytwarzane ilości tych tworzyw stale rosły, a w 2020 roku wyprodukowano 367 milionów ton metrycznych pierwotnego tworzywa sztucznego (PlasticsEurope Market Research Group (PEMRG) / Conversio Market & Strategy GmbH, 2021). Wraz z tendencyjnie wzrastającą ilością generowanych tworzyw sztucznych nasilił się poziom niekontrolowanego zaśmiecania nimi planety. W 2020 roku, w Europie zostało zgromadzone ponad 29 milionów ton metrycznych post-konsumenckich odpadów z tworzyw syntetycznych, z czego 34,6 % zostało poddane recyklingowi. Budującym jest fakt, że w porównaniu z 2020 rokiem, roczna ilość tworzyw sztucznych trafiająca na wysypiska spadła o 6 milionów ton metrycznych (PlasticsEurope Market Research Group (PEMRG) / Conversio Market & Strategy GmbH, 2021).

Jednym z najpowszechniejszych polimerów, wykorzystywanych na szeroką skalę do produkcji opakowań oraz w przemyśle samochodowym, jest poli(tereftalan etylenu) (PET), stanowiący 8,4 % dystrybuowanych polimerów w Europie. Obok niego, najpopularniejszymi typami tworzyw wykorzystywanymi na Starym Kontynencie są: polipropylen (PP)- 19,7 %, polietylen o niskiej i liniowo-niskiej gęstości (PE-LD, -LLD)- 17,4 %, polietylen o wysokiej i średniej gęstości (PE-HD, -MD)- 12,9 %, polichlorek winylu (PVC)- 9,6 %, poliuretan (PUR)- 7,8 % oraz polistyren (PS)- 6,1 % (Chamas et al., 2020; Plastics Europe Market Research Group (PEMRG) and Conversio Market & Strategy GmbH., 2021). Posiadają one dużą masę cząsteczkową, zapewniającą dobre właściwości mechaniczne tworzywa, takie jak: sztywność, elastyczność, odporność na uszkodzenia i pęknięcia, a dodatkowo cechują się wysoką hydrofobowością (Al-Sabagh et al., 2016; Danso et al., 2019). Istotne, pod względem wykorzystania przemysłowego, cechy polimerów syntetycznych przyczyniają się do ich utrudnionej degradacji z wykorzystaniem metod recyklingu biologicznego (Chamas et al., 2020). Dotychczasowe doniesienia wskazują także, że tworzywa sztuczne w środowiskach morskich są związane z trwałymi zanieczyszczeniami organicznymi (POP), takimi jak polichlorowane bifenyle (PCB), pestycydy czy wielopierścieniowe węglowodory aromatyczne (PAH) (Chamas et al., 2020; Fisner et al., 2013; Rios et al., 2010).

Oprócz niekorzystnego wpływu dużych fragmentów odpadów syntetycznych na środowisko, niewątpliwym problemem występującym równolegle jest uwalnianie się w wyniku procesów abiotycznych małych elementów tworzyw sztucznych, tzw. mikro- (1 μm—5 mm) i nanoplastików (<1 μm). Skażenie planety mikroplastikiem rozszerzyło się do tego stopnia, że jego obecność stwierdzona została w środowisku glebowym na terenach rolniczych, przybrzeżnych, przemysłowych (Xu et al., 2020), a także w środowiskach odległych od siedzib ludzkich, takich jak Arktyka (Cózar et al., 2017), Antarktyda (Waller et al., 2017) i Rów Mariański (Gangadoo et al., 2020).

Zanieczyszczenie terenów lądowych oraz morskich śmieciami z tworzyw sztucznych związane jest z niekontrolowanym uwalnianiem odpadów na wysypiska oraz do rzek, skąd przedostają się one dalej do środowisk mórz i oceanów, stanowiąc duże zagrożenie dla organizmów morskich i nadmorskich (Lebreton et al., 2017). Szacuje się, że rocznie do środowisk wodnych trafia 4,8-12,7 milionów ton metrycznych tego typu śmieci (Jambeck et al., 2015). Dotychczas z powodu zanieczyszczenia środowiska tworzywami syntetycznymi śmierć poniosło ponad 1 milion ptaków nadmorskich oraz 100 tysięcy ssaków i żółwi (Jaiswal et al., 2020). Wszechobecność mikroplastików (MP), czyli małych fragmentów tworzyw sztucznych, w środowisku stanowi również zagrożenie dla ludzi. Pofragmentowane materiały polimerowe mogą przedostawać się do organizmu poprzez spożycie lub kontakt skórny, co może powodować potencjalnie długotrwały uszczerbek na zdrowiu (Hirt and Body-Malapel, 2020; Prata et al., 2020). Wnikanie fragmentów mikroplastiku czy nanoplastiku do łańcucha pokarmowego wiąże się z dodatkowymi ryzykiem związanym z możliwością ich akumulacji w jelitach, nerkach czy wątrobie, co świadczy o możliwości przenoszenia drobnych fragmentów tworzyw przez układ krążenia. W ostatnim czasie obecność mikroplastików we krwi ludzkiej została potwierdzona. We krwi 77 % przebadanych osób wykryto PMMA (szkło akrylowe), PP (polipropylen), PS (polistyren), PE (polietylen) oraz PET (poli(tereftalan etylenu) (Leslie et al., 2022). Dotychczasowe badania *in vitro* wskazały, że mikroplastiki mogą indukować odpowiedź immunologiczną, powodują stres oksydacyjny, zmianę integralności błon oraz wpływają na ekspresję genów (Bouwmeester et al., 2015; Mattsson et al., 2017; Meaza et al., 2021). Ekspozycja ludzi na spożycie mikroplastików ma związek z wykryciem ich obecności w wodach powierzchniowych, gruntowych, kranowych oraz ściekach (Novotna et al., 2019; SAPEA Science Advice for Policy by European Academies, 2019). Obecność mikroplastików została także potwierdzona w produktach spożywczych, takich jak miód, cukier (Liebezeit and

Liebezeit, 2013) i krowie mleko (Kutralam-Muniasamy et al., 2020). Ostateczne potwierdzenie faktu spożywania plastiku przez ludzi zostało zdeterminowane po przebadaniu próbek kału, w których ujawniono obecność 9 typów tworzyw sztucznych, a najczęściej występującymi był PP i PET (Raamsdonk et al., 2020). Warto zauważyć, że mikroplastiki o wymiarach przekraczających 150 μm nie są wchłaniane w jelitach, jednak, wiążąc się z warstwą śluzu jelitowego, mogą prowadzić do ich zapalenia i wpływać przez to na układ odpornościowy. Mniejsze fragmenty mogą przekroczyć barierę śluzową i, pomimo mało wydajnego wchłaniania plastiku w jelicie (0,3 %), jak wykazały badania przeprowadzone na zwierzętach, tworzywa polimerowe akumulują się w wątrobie, śledzionie, sercu, płucach, nerkach a nawet w mózgu (Carr et al., 2012; EFSA Panel on Contaminants in the Food Chain, 2016; Prüst et al., 2020).

Badania przeprowadzone w zakresie degradacji tworzyw sztucznych wyodrębniły szereg enzymów z klasy hydrolaz, zdolnych do hydrolizy wiązań estrowych obecnych w materiałach syntetycznych. Enzymy posiadające potencjał degradacyjny tworzyw sztucznych to: kutynazy (EC 3.1.1.74); PETaza (EC 3.1.1.101) (przy współudziale MHETazy (EC 2.1.1.102)) oraz lipazy (EC 3.1.1.3). α/β-hydrolazy o zdolności dekompozycji PET wyizolowano z *Humicola insolens* (HiC), *Thermobifida fusca* (TfCut2), kompostu liściowo-gałęziowego (LCC) (Tournier et al., 2020) (kutynazy) oraz *Ideonella sakaiensis* (PETaza i MHETaza) (Furukawa et al., 2019; Yoshida et al., 2016). Kutynazy są w stanie hydrolizować zarówno wiązania estrowe występujące w poliestrach alifatycznych, jak i aromatycznych, dzięki czemu możliwe jest ich zastosowanie w degradacji szerokiej gamy polimerów sztucznych (Liu et al., 2019; Sulaiman et al., 2012). Z kolei PETaza może hydrolizować wiązania estrowe obecne tylko w poliestrach aromatycznych (Austin et al., 2018). Enzymy biorące udział w degradacji PET należą do podklasy esteraz i posiadają triadę katalityczną charakterystyczną dla α/β-hydrolaz (Ser-His-Asp). Hydroliza wiązania estrowego zachodzi dzięki nukleofilowemu atakowi atomu tlenu seryny na karbonylowy atom węgla obecny w wiązaniu estrowym. Ujemnie naładowany asparaginian (Asp) stabilizuje dodatnio naładowaną resztę histydynową (His), a powstająca sieć przenoszenia ładunku umożliwia serynie (Ser) przeprowadzenie ataku nukleofilowego (Han et al., 2017). Produktami powstającymi podczas degradacji enzymatycznej PET są: BHET (tereftalan bis(2-hydroksyetylu)), MHET (kwas mono-(2-hydroksyetylowy) tereftalowy), TPA (kwas tereftalowy) i EG (glikol etylenowy) (P3; Rys. 1). Do celów niniejszej pracy, na podstawie dotychczasowych doniesień naukowych związanych z wydajnością określonych enzymów

pod kątem degradacji tworzyw sztucznych, wybrano: kutynazy z *Trichoderma reesei* oraz *Fusarium solani*, PETazę z *Ideonella sakaiensis* oraz lipazę 2 z *Yarrowia lipolytica*. Kutynaza z *Trichoderma reesei* ma dwa optima pH (4,0 i 7,3) i utrzymuje aktywność w zakresie pH 3-8 i traci stabilność w temperaturze powyżej 60 °C (Roussel et al., 2014). Kutynaza z *Fusarium solani* wykazuje aktywność w szerokim zakresie pH (3-11), a optymalne pH działania zostało doświadczalnie ustalone w przedziale 6-9. Enzym jest stabilny w zakresie 20-70 °C, przy czym optymalna temperatura działania mieści się w zakresie na 30 i 40 °C (Baker et al., 2012; Chen et al., 2008). PETaza jest enzymem o optimum temperaturowym wynoszącym 40 °C i wykazującym aktywność w pH 6-10 (Chen i in., 2021). Lipaza 2 jest stabilna w pH 3,5-9, natomiast tolerowany zakres temperatur mieści się w przedziale pomiędzy 25 °C a 55 °C. Optymalne warunki działania dla tej hydrolazy zostały eksperymentalnie ustalone na 37 °C i pH 7,0 (Yu et al., 2007).

Rozszerzenie wiedzy dotyczącej enzymów zaangażowanych w procesy degradacyjne tworzyw sztucznych pozwala na udoskonalenie poznanych dotychczas metod z wykorzystaniem inżynierii genetycznej. Do tej pory przeprowadzono wiele modyfikacji genetycznych w celu poprawy naturalnych zdolności mikroorganizmów do rozkładu materiałów syntetycznych (Liu et al., 2019) lub poprzez nadekspresję enzymów zaangażowanych w ten proces w heterologicznym gospodarzu (Gamerith et al., 2017; Ribitsch et al., 2011; Xi et al., 2021). Istotnym etapem pracy związanym z konstruowaniem nowych szczepów w celu zewnątrzkomórkowej produkcji interesujących nas białek, jest dobór szczepu będącego organizmem gospodarza. W badaniach przeprowadzonych w niniejszej pracy, mikroorganizmem wykorzystanym do sekrecji wybranych α/β-hydrolaz były niekonwencjonalne drożdże *Yarrowia lipolytica*. Kryteria doboru, jakie zostały ustalone podczas selekcji organizmu gospodarza, wiązały się z założeniem przeprowadzenia procesu degradacji polimerów bezpośrednio w hodowli mikroorganizmu, przy zapewnieniu odpowiednich warunków środowiskowych oraz z możliwością wykorzystania uwolnionych produktów degradacji jako źródła węgla. Drożdże *Y. lipolytica* posiadają zdolność do asymilacji atypowych źródeł węgla, takich jak alkany czy poliole, mogą rosnąć w szerokim zakresie pH a dodatkowo posiadają status GRAS (generalnie uznane za bezpieczne; ang. Generally Recognised as Safe) (Rzechonek et al., 2020).

2. Cel pracy

Celem pracy było zbadanie zdolności zmodyfikowanych genetycznie drożdży *Yarrowia lipolytica* do degradacji tworzyw sztucznych. Realizacja badań nad powyższym tematem prowadzona była w oparciu o cele szczegółowe:

- Inżynieria genetyczna drożdży *Y. lipolytica* w celu uzyskania szczepów funkcjonalnie ekspresjonujących wybrane geny hydrolaz.
- Określenie zdolności uzyskanych szczepów do rozkładu plastiku z wykorzystaniem poli(ε kaprolaktonu) (PCL) oraz poli(tereftalanu etylenu) (PET) jako substratów modelowych.
- Zweryfikowanie zdolności drożdży do wzrostu na terminalnych produktach degradacji tworzyw sztucznych.
- Optymalizacja procesu degradacji materiałów syntetycznych bezpośrednio w hodowli mikroorganizmów.

3. Materiały i metody

Badania do niniejszej pracy wykonane zostały z wykorzystaniem szczepów *Yarrowia lipolytica* modyfikowanych genetycznie. Poniższy rozdział zawiera informacje dotyczące wykorzystanych mikroorganizmów oraz podstawowych metod użytych podczas pracy laboratoryjnej. Szczegółowe informacje dotyczące zaimplementowanych protokołów znajdują się w publikacjach wchodzących w skład rozprawy doktorskiej.

3.1. Mikroorganizmy

Szczepy drożdży wykorzystane w pracy zostały uzyskane z dzikiego szczepu *Yarrowia lipolytica* A101 (Wojtatowicz and Rymowicz, 1991), w którym dokonano delecji genu dekarboksylazy 5'-fosforanu orotydyny (URA3) (AJD) (Mirończuk et al., 2015) a następnie usunięto proteazy: kwaśną (AXP; YALI0B05654g) oraz zasadową (XPR2; YALI0F31889g) (Janek et al., 2020). Otrzymany w wyniku powyższych modyfikacji szczep AJD ΔXΔA (AJD 2) został wykorzystany do utworzenia szczepów posiadających zdolność do ekspresji genów kutynaz z *Fusarium solani* i *Trichoderma reesei*, PETazy z *Ideonella sakaiensis* a także do nadekspresji natywnej lipazy 2 (YALI0A20350g). Geny kutynaz oraz PETazy wklonowane do *Y. lipolytica* zostały zoptymalizowane kodonowo i posiadają sekwencję sygnalną XPR2 umożliwiającą zewnątrzkomórkową sekrecję wyprodukowanych enzymów (Nicaud et al., 1989). Jako nośnik plazmidów zawierających wyżej wymienione geny wykorzystano bakterię *Escherichia coli* DH5α. Szczepy wykorzystane w badaniach zostały przedstawione w Tabela 1.

Tabela 1. Zestawienie szczepów wykorzystanych podczas pracy laboratoryjnej.

Szczep	Genotyp	Źródło
	Escherichia coli	
DH5α	F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^-m_K^+$), λ$^-$	(Hanahan and Glover, 1985)
pAD Lip2	Plazmid niosący gen YALI0A20350g pod promotorem UAS1B16- TEF, YALI0A20350g, *amp^r*	(Janek et al., 2020)

pAD CUT_FS	Plazmid niosący zoptymalizowany kodonowo gen kutynazy z *Fusarium solani* pod promotorem UAS1B16-TEF, *amp*^{r}	(Kosiorowska et al., 2021)
pAD CUT_TR	Plazmid niosący zoptymalizowany kodonowo gen kutynazy z *Trichoderma reseei* pod promotorem UAS1B16- TEF, *amp*^{r}	
pAD PET_IS	Plazmid niosący zoptymalizowany kodonowo gen PETazy z *Ideonella sakaiensis* pod promotorem UAS1B16- TEF, *amp*^{r}	(Kosiorowska et al., 2022)
Yarrowia lipolytica		
A101	Wild type	(Wojtatowicz and Rymowicz, 1991)
AJD	MATA, A101: ura3-302	(Mirończuk et al., 2015)
AJD 2	MATA, A101, ΔAXP, ΔXPR2	(Janek et al., 2020)
AJD 2 pAD Lip2	Nadekspresja YALI0A20350g	
AJD 2 pAD CUT_FS	Ekspresja kutynazy z *Fusarium solani*	(Kosiorowska et al., 2021)
AJD 2 pAD CUT_TR	Ekspresja kutynazy z *Trichoderma reesei*	
AJD 2 pAD Lip2/CUT_FS	Nadekspresja lipazy 2 (YALI0A20350g) i kutynazy z *Fusarium solani*	
AJD 2 pAD Lip2/CUT_TR	Nadekspresja lipazy 2 (YALI0A20350g) i kutynazy *Trichoderma reesei*	
AJD 2 pAD PET_IS	Ekspresja PETazy z *Ideonella sakaiensis*	(Kosiorowska et al., 2022)

3.2. Podłoża i warunki hodowli

Bakterie *E. coli* hodowano w podłożu LB w temperaturze 37 °C przy ciągłym wstrząsaniu 200 RPM. Bakterie niosące plazmidy, hodowane były w podłożu selekcyjnym LB z dodatkiem ampicyliny w stężeniu końcowym 100 μgmL^{-1}. Drożdże każdorazowo wzrastały w temperaturze 28 °C przy ciągłym wstrząsaniu 200 RPM. Inokulum *Y. lipolytica* podłoża oraz hodowle z dodatkiem 0,5 g folii z PCL oraz krzywe wzrostu szczepów drożdżowych hodowano w podłożu YPD (1 % ekstrakt drożdżowy, 2 % pepton, 2 % glukoza). Do badania profilu wzrostu drożdży wykorzystano czytnik płytek Spark Tecan (Männedorf, Szwajcaria), gęstość optyczną hodowli mierzono przy długości fali 600 nm co 30 min przy ciągłym mieszaniu orbitalnym. Do izolacji RNA wykorzystano podłoże YNB (Yeast Nitrogen Base, podłoże syntetyczne, Sigma Aldrich) z 2 % zawartością glukozy. Testy stref przejaśnień przeprowadzone w Publikacji 1 wykonane zostały na stałym podłożu YNB z 0,1 % dodatkiem zemulsyfikowanego poli(ε-kaprolaktonu) (PCL) i 2 % agaru. Weryfikację zdolności degradacji proszku i folii PET (GoodFellow, ES306031/1; ES301445/7, Wielka Brytania) przeprowadzono w podłożu YPD z 5 % zawartością glukozy i dodatkiem odpowiednio 2 g i 1 g materiału plastikowego. OD startowe drożdży w testach sprawdzających profil wzrostu na płytkach 96- dołkowych wynosiło zawsze 0,05, dla hodowli prowadzonych w płytkach głębokodołkowych 0,1 natomiast w przypadku hodowli w 0.3 L kolbach Erlenmeyera 0,5 (dla tworzywa PET) lub 1 (dla tworzywa PCL). Określenie gęstości hodowli inokularnej oznaczano metodą turbidymetryczną poprzez mierzenie gęstości optycznej hodowli przy λ= 600 nm z zachowaniem liniowości otrzymywanych odczytów z użyciem spektrofotometru UV-vis (BIO-RAD, USA). Szczegółowe dane dotyczące podłóż selekcyjnych oraz protokołu przygotowania podłóż suplementowanych zawarte zostały w odpowiednich publikacjach.

3.3. Modyfikacje genetyczne mikroorganizmów

Inżynieria metaboliczna drożdży wykonana w ramach niniejszej pracy opierała się na wykorzystaniu bakterii *E. coli* jako nośnika skonstruowanych plazmidów zawierających wybrane geny oraz późniejszej transformacji *Y. lipolytica* w celu zintegrowania genów w genomie drożdży. Heterologiczne geny zostały zoptymalizowane kodonowo, poddane fuzji z sekwencją sygnalną XPR2 oraz zsyntezowane w laboratorium komercyjnym (Sigma Aldrich, GeneWizzaird, Wielka Brytania).

3.3.1. Transformacja *E. coli*

Komórki kompetentne bakterii *E. coli* przygotowywano zgodnie z protokołem metody chemicznej z wykorzystaniem chlorku wapnia ($CaCl_2$) opisanego wcześniej (Hanahan et al., 1991). Bakterie transformowano metodą szoku cieplnego. Plazmidy niosące geny kutynaz z *F. solani* oraz *T. reesei* oraz PETazy z *I. sakaiensis* skonstruowano w oparciu o wektor pAD (Mirończuk et al., 2017) z wykorzystaniem enzymów restrykcyjnych *SgsI* oraz *NheI* oraz ligazy T4 (Thermo Fisher Scientific). Trawienie restrykcyjne oraz ligacja zostały przeprowadzone zgodnie z protokołem producenta. W celu weryfikacji poprawności otrzymanych konstruktów, plazmidy wyizolowane z wykorzystaniem Kit-u Plasmid Mini (A&A Biotechnology, Polska) zostały zsekwencjonowane (Genomed, Polska).

3.3.2. Transformacja *Y. lipolytica*

Transformacja drożdży *Y. lipolytica* przeprowadzona została z użyciem metody wykorzystującej octan litu (LiAc) (Schiestl and Gietz, 1989). Skonstruowane wektory zostały zlinearyzowane przez zastosowanie enzymu restrykcyjnego *MssI* a następnie wklonowane do drożdży w oparciu o metodę rekombinacji z rDNA (rekombinacyjne DNA). Transformanty wzrastały na podłożu YNB z 2 % glukozą. Z wygenerowanych szczepów wyizolowano genomowe DNA używając Genomic Mini AX Yeast Spit Kit (A&A Biotechnology, Polska) i zweryfikowano poprawność otrzymanych mutantów w oparciu o standardową reakcję PCR. Ze względu na użycie genu markerowego URA3 jako czynnika selekcyjnego, w szczepach z podwójną ekspresją genów konieczne było przeprowadzenie procesu przywracania auksotrofii względem uracylu. Proces ten odbywał się z wykorzystaniem tej samej metody transformacji drożdży, jednakże zamiast kasety ekspresyjnej stosowano plazmid pUB4-Cre1 a system użyty w tym protokole był oparty na rekombinazie Cre-Lox (Fickers et al., 2003). Komórki poddane transformacji wysiewano na podłoże selekcyjne YPD z dodatkiem 0,25 gL^{-1} Higromycyny B a następnie pasażowano trzykrotnie na podłoże YPD. Weryfikacja poprawności delecji genu URA3 odbywała się z wykorzystaniem podłoża YNB z 2 % glukozą, na którym poprawne szczepy nie posiadały możliwości wzrostu.

3.4. Sprawdzanie poziomu ekspresji genów (qRT-PCR)

Skonstruowane szczepy drożdży zostały zweryfikowane pod kątem funkcjonalnej ekspresji wklonowanych genów z użyciem metody qRT-PCR. RNA izolowano z hodowli 24 godzinnych z wykorzystaniem Kitu Total RNA Mini Plus (A&A Biotechnology, Polska),

następnie traktowano je DNAzą wolną od rybonukleaz. Synteza cDNA (komplementarne DNA) została wykonana z użyciem Maxima First Strand Synthesis Kit (Thermo Fisher Scientific). Do analizy qRT-PCR wykorzystano zestaw DyNAmo Flash SYBR Green qPCR. Eksperymenty i analiza danych odbywała się z użyciem sprzętu CFX Connect i oprogramowania CFX Maestro (BIO-RAD, USA).

3.5. Analiza filogenetyczna białek

Analiza sekwencji domen należących do rodziny α/β-hydrolaz w zidentyfikowanych dotychczas kutynazach i lipazach została wykonana z wykorzystaniem współrzędnych domen zaczerpniętych z Conserved Domain Database (Lu et al., 2020). W przypadku PETazy, której nie ustalono dotychczas dokładnej pozycji domeny, użyto całej sekwencji aminokwasowej enzymu. Program COBALT (Papadopoulos and Agarwala, 2007) został użyty do wyrównania sekwencji a wizualizacja danych została przeprowadzona w CLC Sequence Viewer 8.0 (QIAGEN). Konstrukcja drzewa filogenetycznego opracowana została metodą najbliższego sąsiada (NJ, ang. Neighbor Joining), odległość pomiędzy badanymi sekwencjami obliczona została z użyciem modelu Jukesa-Cantora po oszacowaniu wiarygodności metodą samopróbkowania (ang. bootstrap) na poziomie 1000 przyrównań sekwencji.

3.6. Badanie aktywności enzymatycznej

Aktywność enzymatyczna esteraz wyprodukowanych przez szczepy została określona na podstawie hydrolizy octanu 4-nitrofenolu. Reakcje enzymatyczne były prowadzone w 50 mM buforze fosforanowym o pH 7.0 oraz 9.0. Substrat przygotowany został w stężeniu 100 mM poprzez rozpuszczenie w DMSO (dimetylosulfotlenek). Mieszaninę bufor i octan 4-nitrofenolu przygotowywano bezpośrednio przed pomiarem. Kinetyczny pomiar absorbancji prowadzono w temperaturze 37 °C przez 10 minut przy λ= 405 nm z wykorzystaniem czytnika mikropłytek Synergy H1 (BIO-RAD, USA). Jedna jednostka enzymu hydrolizuje 1,0 μmola octanu 4-nitrofenolu do kwasu octowego i 4-nitrofenolu w ciągu minuty.

3.7. Warunki prowadzenia procesu degradacji tworzyw sztucznych

3.7.1. Degradacja PCL

Folie PCL przygotowano przez rozpuszczenie granulek poli(ε-kaprolaktonu) w dichlorometanie (Urbanek et al., 2017). Tak przygotowaną mieszaninę przeniesiono na szklane płytki Petriego w celu odparowania rozpuszczalnika. Wysuszone folie PCL zostały

pocięte na fragmenty o masie 0,5 g i wysterylizowane poprzez płukanie w 96 % etanolu i poddane 30-minutowemu naświetlaniu promieniami UV. Hodowle drożdży prowadzone były w 0,3 L kolbach Erlenmeyera w 30 mL podłoża YPD i dodatkiem 0,5 g folii PCL. Próbki hodowli przeznaczone do analizy ilości uwolnionego produktu rozkładu pobierane były co 24 h hodowli przez 72 h. Hodowle z których pozyskiwano materiał do weryfikacji ubytku masy folii PCL, prowadzone były w takich samych warunkach przez 144 h. Próbki pobierane z hodowli były wirowane przy 21 000 G przez 10 minut w temperaturze pokojowej, supernatant przenoszono do nowej probówki typu Eppendorf a następnie mrożono w -20 °C.

3.7.2. Degradacja PET

Zdolność zmodyfikowanych szczepów do degradacji materiału PET oceniono przy użyciu hodowli na płytkach głębokodołkowych (DWP). Szczep drożdży produkujący kutynazę z *F. solani*, PETazę z *I. sakaiensis* oraz szczep kontrolny AJD 2 kultywowano w 4 ml podłoża YPD z 5 % glukozą przez 168 godzin. Próbki pobierano codziennie, począwszy od 72 h hodowli. Pobrane próby wirowano przy 10 000 G, przenoszono supernatant a następnie mrożono w -20 °C. Każda hodowla zawierała 0,266 g sproszkowanego poli(tereftalanu etylenu) (PET) (półkrystaliczny; 300 μm; GoodFellow ES306031/1, Anglia), uprzednio wysterylizowanego przez 10-minutowe promieniowanie UV. Proszek PET dodawany był do hodowli bezpośrednio przed inokulacją podłoża. Hodowle w małej skali laboratoryjnej wykorzystywano również w celu skriningu suplementów mogących zwiększać wydajność degradacji tworzywa sztucznego. Uprzednio wysterylizowane poprzez filtrację z użyciem 0,22 μm filtrów strzykawkowych (Merck Millipore) sole dodawano do podłoża YPD z 5 % zawartością glukozy. Sprawdzono wpływ dodatku: jednowodnego siarczanu (VI) manganu (II)- $MnSO_4 \cdot H_2O$, siedmiowodnego siarczanu (VI) cynku (II)- $ZnSO_4 \cdot 7H2O$, pięciowodnego siarczanu (VI) miedzi (II)- $CuSO_4 \cdot 5H2O$, chlorku wapnia- $CaCl_2$, siedmiowodnego siarczanu (VI) magnezu- $MgSO_4 \cdot 7H2O$ w stężeniach końcowych 1 mM i 2,5 mM. Sole dodawano do hodowli z roztworów skoncentrowanych o stężeniu 50 mM, a końcowe stężenie suplementu obliczano z uwzględnieniem hydratowości wykorzystanych związków. W badaniach przesiewowych wykorzystano również oliwę z oliwek i olej rzepakowy, które zostały uprzednio wysterylizowane poprzez autoklawowanie.

Eksperymenty przeprowadzone w celu określenia zdolności zmodyfikowanych szczepów drożdży do degradacji tworzywa PET w większej skali laboratoryjnej w kolbach

Erlenmeyera o pojemności 0,3 L. Doświadczenia wykonane z użyciem hodowli wstrząsanych w kolbach prowadzono przez 240 h hodowli w 30 mL podłoża YPD z 5 % zawartością glukozy i 2 g proszku PET uprzednio wysterylizowanego przez 10 minut promieniowaniem UV. Tworzywo sztuczne dodawano na początku hodowli. Próbki pobierano codziennie

od 72 h hodowli.

Doświadczenia prowadzone ze szczepem AJD 2 pAD CUT_FS wykonane w dużej skali laboratoryjnej zostały zrealizowane w reaktorze ze zbiornikiem mieszadłowym o objętość całkowitej 5 L (BIOSTAT B-PLUS, Sartorius, Niemcy). Badania w bioreaktorze prowadzono w objętości roboczej 1,0 L w podłożu YPD z 5 % zawartością glukozy w temperaturze 28 °C. Szybkości napowietrzania i mieszania ustalono na 0,8 objętości na minutę (vvm) i 700 min^{-1}. Wartość pH na poziomie 8,5 utrzymywano automatycznie przez dodanie 20 % wodorotlenku sodu (NaOH) i 10% kwasu chlorowodorowego (HCl). W razie potrzeby automatycznie dodawano roztwór środka przeciwpieniącego (BEKCHEM S.C., Polska). Proszek PET, uprzednio wysterylizowany przez 10 min naświetlania promieniami UV, dodano do hodowli po 48 h kultywacji. Próbki pobierano codziennie, począwszy od 72 h trwania hodowli. Czas trwania hodowli wynosił 240 h, co odpowiada 192 h bezpośredniego rozkładu proszku PET w hodowli.

Hodowle drożdży z dodatkiem folii PET jako substratu wykonano w 0,3 L kolbach Erlenmeyera w 50 mL podłoża YPD z 5 % zawartością glukozy i prowadzono przez 4 tygodnie. Do doświadczeń wykorzystano przezroczystą, amorficzną folię PET o grubości 0,25 mm (GoodFellow ES301445/7, Anglia). Folię PET pocięto wcześniej na fragmenty o wymiarach 1 cm× 1 cm i sterylizowano przez 10 min za pomocą promieniowania UV. Folie pobrane po hodowli zostały dwukrotnie przemyte wodą Mili-Q w celu usunięcia biomasy drożdży i pozostawione do wyschnięcia w temperaturze pokojowej.

Hodowle wykonane z wykorzystaniem płytek głębokodołkowych, 0,3 L kolbach Erlenmeyera i bioreaktorach przeprowadzono w trzech powtórzeniach biologicznych. Ilość proszku PET dodawanego do hodowli została ustalona na podstawie badań przedwstępnych pozwalających na ogólne zoptymalizowanie warunków hodowli pod kątem ich przygotowania.

3.8. Analiza chromatograficzna analitów obecnych w płynie pohodowlanym

3.8.1. Wysokosprawna chromatografia cieczowa (HPLC)

Oznaczenie ilości glukozy, glikolu etylenowego (EG) oraz ε-kaprolaktonu została przeprowadzona z użyciem wysokosprawnej chromatografii cieczowej (HPLC). W tym celu wykorzystano UltiMate 3000 UHPLC (Thermo Fisher Scientific, Waltham, USA) oraz kolumnę HyperRez CarbH + z detektorem UV (ʎ = 210 nm) (Dionex, Sunnyvale, USA) i RI (Shodex, Ogimachi, Japonia). Jako eluentu użyto 25 mM kwasu trifluorooctowego (TFA). Temperaturę kolumny ustawiono na 65 °C, a szybkość przepływu wynosiła 0,6 ml min^{-1}. Wzorce glukozy i EG zostały przygotowane przez rozcieńczenie analitów w wodzie Mili-Q, natomiast ε-kaprolakton przygotowano przez rozpuszczenie w metanolu (Chempur, Polska). Próbki do analizy ilości glukozy oraz EG przygotowano poprzez rozcieńczenie supernatantów w wodzie miliQ, natomiast poziom uwolnionego produktu rozkładu PCL przygotowano przez rozcieńczenie w 99 % metanolu. Stężenie glukozy, EG oraz ε-kaprolaktonu analizowano w programie Chromeleon Chromatography Data System (Thermo Fisher Scientific, Waltham, USA).

3.8.2. Ultrasprawna chromatografia cieczowa (UHPLC)

Analiza jakościowa i ilościowa supernatantu pochodzącego z procesu degradacji PET została przeprowadzona z użyciem ultrasprawnej chromatografii cieczowej (UPLC) przy użyciu systemu UltiMate 3000 UHPLC (Thermo Fisher Scientific, Waltham) z zastosowaniem kolumny typu C18 (Hypersil Gold 100 × 2,1 mm, 3 μm) z uniwersalną kolumną ochronną (Hypersil Gold 10 × 2,1 mm, 3 μm). Do gradientowej elucji użyto dwóch faz ruchomych: (A) acetonitryl z 0,1 % kwasem trifluorooctowym (v/v) i (B) H_2O z 0,1% kwasem trifluorooctowym (v/v). Szybkość przepływu wynosiła 0,8 $mLmin^{-1}$, objętość nastrzyku 1 μL, a temperatura kolumny 45 °C. Detekcja analitów odbywała się za pomocą detektora UV (Dionex, Sunnyvale, USA) przy długości fali 243 ± 2 nm. Tożsamość analitów potwierdzono dodatkowo analizą widma absorpcyjnego w zakresie 190-500 nm. Użyta metoda pozwala na całkowite rozdzielenie analitów w ciągu 7 min przy maksymalnym ciśnieniu 250 barów. Próbki pobrane z doświadczeń przeprowadzonych w małej skali laboratoryjnej, w 0,3 L kolbach Erlenmeyera oraz bioreaktorach odwirowywano przy 10 000 G przez 10 min. Supernatanty rozcieńczano w 99,9% metanolu i odwirowywano przez 10 minut przy 10 000 G w temperaturze 10 °C. Roztworami wzorcowymi wykorzystane w analizie produktów degradacji PET były: roztwór kwasu tereftalowego (TPA) (CAS: 100-21-0, Sigma Aldrich) roztwór kwasu mono(2-hydroksyetylo)tereftalowego (MHET) (CAS:

1137-99-1, AChemBlock) przygotowanych poprzez rozpuszczenie w dimetylu sulfotlenku (DMSO), i roztwór tereftalanu bis(2-hydroksyetylu) (BHET) (CAS: 959-26-2, Sigma) przez rozpuszczenie w 99,9 % metanolu. Wzorce o stężeniu końcowym 10 gL^{-1} przechowywano w zamrażarce w temperaturze -20 °C. Krzywe standardowe sporządzono na podstawie roztworów wzorcowych przez odpowiednie rozcieńczenie analitów w 99,9% metanolu. Analizę danych przeprowadzono przy użyciu programu Chromeleon 7.1.

3.8.3. Analiza statystyczna wyników

Wyniki uzyskane z użyciem metod chromatograficznych zostały poddane analizie statystycznej w celu określenia istotności wpływu zastosowanych dodatków na ilość uwolnionych produktów rozkładu. Analizę przeprowadzono przy użyciu testu t-Studenta, poprzez porównanie średnich wyników uzyskanych w hodowli kontrolnej (bez dodatku czynnika badanego) wobec konkretnej hodowli suplementowanej (test dwuwarstwowy; o nierównej wariancji; $*p \leq 0,5$, $**p \leq 0,01$, $***p \leq 0,005$).

3.9. Skaningowa mikroskopia elektronowa (SEM)

W celu określenia zmian morfologii powierzchni podczas degradacji PET w hodowlach ze zmodyfikowanymi szczepami drożdży *Y. lipolytica*, wykorzystano skaningowy mikroskop elektronowy (SEM) VEGA Tescan 3. Próby folii pobrane po hodowli zostały poddane napylaniu cienkiej warstwy złota, w celu uniknięcia gromadzenia się nadmiernego ładunku elektrycznego. Warunkami napylania użytymi w tym procesie było: natężenie prądu 40 mA, czas napylania: 60 s (Cressington, Sputter Coater 108). Mikrografy SEM otrzymano przy użyciu mikroskopu VEGA Tescan 3 i dedykowanego oprogramowania.

4. Komentarze do publikacji

4.1. **Publikacja 1- Urbanek, A.K., Kosiorowska, K.E., Mirończuk, A.M., 2021. Current Knowledge on Polyethylene Terephthalate Degradation by Genetically Modified Microorganisms. Front. Bioeng. Biotechnol. 9, 1–15.**

W Publikacji 1 został szczegółowo opisany aktualny stan wiedzy związanej z procesem degradacji poli(tereftalanu etylenu), ze szczególnym uwzględnieniem organizmów zmodyfikowanych genetycznie, będącej rozszerzonym wstępem teoretycznym do niniejszej pracy doktorskiej.

Wstęp Publikacji 1 zawiera kluczowe informacje przedstawiające skalę problemu zanieczyszczenia środowiska odpadami tworzyw sztucznych. Zwrócona została uwaga na zagrożenie dla fauny i flory morskiej i nadmorskiej przez uwalniane mikro- i nano-plastiki, które akumulowane przez organizmy mogą powodować szereg zaburzeń, takich jak indukowanie odpowiedzi immunologicznej oraz zmianę integralności błon i ekspresji genów. Częściowo problem nagromadzenia odpadów PET jest rozwiązywany przez recykling skutkujący wytworzeniem nowego PET lub odzyskanie składników służących do produkcji tego materiału tj. TPA i EG. W związku z ograniczeniami powstającymi podczas recyklingu PET, takimi jak konieczność zastosowania wysokiej temperatury i katalizatorów na bazie metali i cieczy jonowych, procesy te są wypierane przez metody biologiczne. Określenie zdolności do rozkładu PET przez enzymy z grupy hydrolaz, takich jak PETaza z *Ideonella sakaiensis* czy kutynaz z *Fusarium solani*, *Humicola insolens* czy *Thermobifida cellulosilytica* otworzyło nowe możliwości w zakresie zarządzania odpadami PET. Jednakże, pomimo wiedzy na temat wielu enzymów, istnieje wiele nierozwiązanych problemów dotyczących ich praktycznego wykorzystania do degradacji PET, takich jak niska stabilność termiczna lub problem z przeniesieniem procesu degradacji na skalę przemysłową. W odpowiedzi na te problemy, zaczęto rozważać wykorzystanie inżynierii białka, aby przezwyciężyć obecne ograniczenia.

W Publikacji 1 przedstawiono dogłębną analizę enzymów poddanych modyfikacjom oraz wpływ zastosowanych zmian na efektywność degradacji PET (P1; Tabela 1). Możliwość wprowadzenia zmian w strukturze białka wiąże się z koniecznością dogłębnego poznania struktury danego enzymu oraz zrozumienie zasady działania poszczególnych reszt aminokwasowych oraz mechanizmu ich działania w procesie hydrolizy wiązań estrowych w procesie degradacji PET.

Zaproponowane dotychczas zmiany w budowie białka PETazy z *I. sakaiensis* dotyczyły głównie modyfikacji w obrębie miejsca aktywnego, polegającego na zmianie argininy w miejscu 280 (R280) na alaninę (A) w celu zwiększenia kieszeni wiążącej substrat oraz zamianę seryny w miejscu 121 (S121) na kwas asparaginowy (D) lub glutaminowy (E) wraz ze zmianą kwasu asparaginowego (D) na histydynę (H), co miało na celu wprowadzenie do cząsteczki dodatkowych wiązań wodorowych skutkujących zwiększeniem stabilności enzymu. Interesującym rozwiązaniem opisanym w Publikacji 1 jest zastosowanie narzędzia Premuse, które przewiduje prawdopodobne zmiany struktury białka wynikające z naturalnej ewolucji łańcucha aminokwasowego. Opisane w pracy mutanty uzyskane z wykorzystaniem tej metody uzyskały 40-krotny wzrost ilości produktów degradacji w porównaniu z enzymem natywnym.

Badania prowadzone w celu zweryfikowania mechanizmu degradacji PET przez PETazę produkowaną przez bakterię *I. sakaiensis* doprowadziły do odkrycia innego enzymu współodpowiedzialnego za całkowitą hydrolizę PET- MHETazy. Inżynieria metaboliczna MHETazy nie jest jeszcze tak zaawansowana jak w przypadku PETazy, jednakże w pracy wskazano kilka przykładów przeprowadzonych modyfikacji struktury tego białka, skutkujących zwiększoną efektywnością degradacji MHET, będącego jednym z głównych, lecz nieterminalnych produktów rozkładu PET. Jedna z pierwszych mutagenez MHETazy została przeprowadzona w trakcie prac nad określeniem jej dokładnej struktury i dotyczyła zmiany aminokwasów w obrębie miejsca aktywnego enzymu. Przeprowadzone badania ujawniły, że jednym z kluczowych aminokwasów odpowiedzialnych za wiązanie substratów jest fenyloalanina w pozycji 495 (F495), którego zastąpienie alaniną (A) spowodowało powstanie wariantu białka F495A. Badania właściwości katalitycznych tego mutanta wykazały, że szybkość obrotu MHET w porównaniu z enzymem typu dzikiego była ponad 2-krotnie niższa i wynosiła około 5 s^{-1}.

Szeroki zakres badań modyfikacji struktury białek zdolnych do hydrolizy wiązań obecnych w PET został przeprowadzony na kutynazach. Jedna z zastosowanych zmian w łańcuchu aminokwasowym dotyczy kutynazy z *Thermobifida cellulosilytica* (Thc_Cut2), w której spośród 8 uzyskanych mutantów znaczący przyrost ilości produktów rozpadu zanotowano dla Ala30Val, Arg29Asn_Ala30Val i Arg19Ser_Arg29Asn_Ala30Val. Wyniki doświadczeń przybliżonych w Publikacji 1 dotyczące zmian przeprowadzonych w kutynazach wskazały również, że nie każda modyfikacja struktury białka, pomimo zachowanych parametrów kinetycznych mutanta, powoduje zwiększenie wydajności degradacji PET.

W pracy opisano również badania przeprowadzone z użyciem kutynazy z *F. solani*, w których wykonano mutagenezę ukierunkowaną w regionie w pobliżu miejsca aktywnego. Wygenerowane w ramach cytowanej pracy mutanty L81A i L182A wykazały odpowiednio cztero- i pięciokrotny wzrost aktywności wobec włókien PET w porównaniu z enzymem niezmodyfikowanym. Wprowadzona zmiana w strukturze tej kutynazy skutkująca zwiększoną aktywnością wobec PET jest związana z poprawą stabilności białka oraz lepszą zdolnością do wiązania substratu.

W artykule przeglądowym wchodzącym w skład rozprawy doktorskiej przytoczono również możliwość zastosowania białek fuzyjnych, które działając synergicznie wykorzystują cechy dwóch enzymów. Jednym z ciekawych rozwiązań, mających na celu eliminację problemu związanego z powstawaniem MHET, który jest jednym z produktów częściowej hydrolizy PET, było utworzenie chimery białek kutynazy z *Thermobifida fusca* (TfCut2) oraz LC-kutynazy (LCC). W innych badaniach kutynaza z *Thielavia terrestris* NRRL 8126 została połączona z lipazą z *Thermomyces lanuginosus*, a nowe białko fuzyjne zostało wklonowane do *Pichia pastoris*. Tworzywem modelowym na którym weryfikowano efektywność utworzonej dwufunkcyjnej lipazy-kutynazy był PCL. Ubytek masy tworzywa przy zastosowaniu utworzonej chimery był znacząco wyższy niż ten w przypadku użycia pojedynczych enzymów lipazy i kutynazy a także ich mieszaniny. Przeprowadzona analiza SEM folii PCL po traktowaniu chimerą Lip-Cut wykazała, że powierzchnia folii PCL stała się bardziej chropowata i zaobserwowano więcej otworów po 4 h traktowania enzymem dwufunkcyjnym niż w przypadku innych enzymów po 48 h.

Podsumowując, w Publikacji 1 przedstawiony został problem zanieczyszczenia środowiska odpadami plastikowymi oraz opis dotychczasowych doniesień na temat biologicznych metod degradacji PET, co tłumaczy znaczenie badań, jakie zostały podjęte w ramach niniejszej rozprawy doktorskiej.

4.2. **<u>Publikacja 2-</u> Kosiorowska, K.E., Połomska, X., Wang, G., Borodina, I., Mirończuk, A.M., 2021. Efficient biodegradation of aliphatic polyester by genetically engineered strains of the yeast *Yarrowia lipolytica*. Int. Biodeterior. Biodegrad.. Volume 161, July 2021, 105232**

Publikacja ta jest pierwszą pracą z cyklu badań nad zdolnością zmodyfikowanych drożdży *Yarrowia lipolytica* do rozkładu tworzyw sztucznych. W pracy po raz pierwszy

opisano szczepy niekonwencjonalnych drożdży *Y. lipolytica* produkujących zewnątrzkomórkowo kutynazy z *Fusarium solani* (CUT_FS) i *Trichoderma reesei* (CUT_TR) oraz mutantów posiadających koekspresję tych genów z natywną lipazą 2 (Lip2). Przeprowadzona w ramach badań bioinformatycznych analiza filogenetyczna enzymów użytych w pracy z 11 innymi α/β-hydrolazami ujawniła najbliższą homologię białka lipazy 2 z *Y. lipolytica* (AFH77825) z PETazą z *Ideonella sakaiensis* (6EQD_A), podczas gdy kutynaza z *T. reesei* (ETS02914) wykazywała wysoką homologię (53,49 % identyczności) z kutynazą z *Monilinia fructicola* (AAZ95012) natomiast kutynaza z *F. solani* (AAA33334) jest blisko spokrewniona (55,33 % identyczności) z kutynazą z *Humicola insolens* (4OYY_A) (P2; Rys. 1A). Przedstawiona w Publikacji 2 (Rys. 1B) analiza porównawcza wybranych sekwencji enzymów zwraca również uwagę na obecność wysoko konserwatywnego regionu we wszystkich badanych białkach złożony z 5 aminokwasów. Funkcjonalność wygenerowanych w ramach badań szczepów zweryfikowana z użyciem metody qRT-PCR wykazała, że każdy z utworzonych mutantów jest zdolny do ekspresji wklonowanych genów (P2 Rys. 2A). Dodatkowo mutanty użyte w eksperymentach nie przejawiają opóźnień we wzroście w porównaniu ze szczepem kontrolnym AJD 2 (P2; Fig 2B).

W pracy wykorzystano jako tworzywo modelowe poli(ε-kaprolaktonu) (PCL), który jest biodegradowalnym tworzywem sztucznym wykazującym wiele cech wspólnych z PET (poli(tereftalanem etylenu)). Zdolność do degradacji PCL przez uzyskane szczepy drożdży oraz określenie warunków pH optymalnych do prowadzenia hydrolizy wiązań estrowych w wybranym tworzywie modelowym przeprowadzono z użyciem spot-testów w trzech różnych wariantach pH (4,0; 7,0; 9,0) w podłożu zawierającym 0,1 % PCL. Rysunki, na których widać strefy przejaśnień przedstawione w Publikacji 2 (Rys. 3) wskazują, że w pH kwaśnym jedynymi szczepami wykazującymi aktywność hydrolityczną jest AJD 2 pAD CUT_TR oraz podwójny mutant AJD 2 pAD Lip2_CUT_TR, co jest zgodne z wcześniejszymi doniesieniami dot. aktywności kutynazy z *T. reesei*. Te same szczepy są również aktywne w pH 7.0, jednakże w tych warunkach wyróżniająco efektywniejsze wykazały się mutanty produkujące kutynazę z *F. solani* oraz wariant podwójny AJD 2 pAD Lip2_CUT_FS. W warunkach neutralnego pH można również zauważyć strefę przejaśnień pojawiającą się w przypadku zastosowania szczepu AJD 2 pAD Lip2, który wykazuje aktywność również w pH 9.0. W alkalicznym pH również zaobserwowano strefy przejaśnień tworzone przez szczepy produkujące kutynazę z *T. reesei* oraz szczep AJD 2 pAD Lip2_CUT_FS, choć były one mniejsze niż w pH 7,0. Szczep z pojedynczą ekspresją genu

CUT_FS wykazał podobną wielkość strefy przejaśnień do tej zaobserwowanej w pH 7,0. Następnie by potwierdzić aktywność enzymatyczną esteraz, przeprowadzono analizę aktywności enzymatycznej supernatantów zawierających wyprodukowane zewnątrzkomórkowo enzymy kutynaz i lipazy. Wyniki przedstawione na Rys. 4 w Publikacji 2 wskazały, że w przypadku obu kutynaz lepszą zdolność do hydrolizy wiązań estrowych posiadają w pH 9,0. Lipaza 2 z kolei wyższą aktywność uzyskała w pH 7,0 i była ona na poziomie 3,66 UmL^{-1}. Co ciekawe, aktywność enzymatyczna szczepów produkujących kutynazę z *T. reesei* była wyższa w pH 9,0, a mutant z podwójną ekspresją tego genu z Lip2 osiągnął niższy wynik niż szczep AJD 2 pAD CUT_TR. Ten wynik może mieć związek z niższą ekspresją genu CUT_TR w szczepie koekpresjonującym Lip2, związanej z ograniczoną pulą czynnika transkrypcyjnego w komórce dla genów znajdujących się pod tym samym promotorem hybrydowym $UAS1B_{16}$-TEF. Przy zmniejszonej aktywności Lip2 w podanych warunkach sumaryczny wpływ obu enzymów obecnych w supernatancie na hydrolizę PCL jest adekwatnie mniejszy i ma on bezpośredni związek z ilością enzymu CUT_TR obecnego w płynie pohodowlanym. Wyróżniającą aktywność enzymatyczną zmierzoną podczas badań była ta określona w supernatantach szczepów AJD 2 pAD CUT_FS oraz AJD 2 pAD Lip2_CUT_FS wynosząca odpowiednio 63 UmL^{-1} oraz 63,5 UmL^{-1}.

Hodowle zmodyfikowanych szczepów *Y. lipolytica* zostały przebadane pod kątem zdolności do degradacji folii PCL bezpośrednio w hodowli mikroorganizmów. Efektywność degradacji plastiku została określona na podstawie ubytku masy folii podczas 144 h hodowli drożdży z wykorzystanym polimerem oraz ilości uwolnionego ε-kaprolaktonu podczas 72 godzinnej kultywacji. Zgodnie z wynikami z wcześniejszej części pracy, najlepszą efektywność degradacji PCL uzyskano w hodowli ze szczepami AJD 2 pAD CUT_FS oraz AJD 2 pAD Lip2_CUT_FS, w której procentowy ubytek masy plastiku został zmierzony na poziomie przekraczającym 90 %. Z kolei w próbach wykorzystujących AJD 2 pAD CUT_TR oraz AJD 2 pAD Lip2_CUT_TR zmierzony spadek wagi wynosił odpowiednio 9,8 % i 15,8 %. Ilość PCL kontrolowana co 24 h we wszystkich hodowlach wykazała zmienność w poziomie tego analitu w zależności od długości hodowli. Najwyższa zmierzona ilość ε-kaprolaktonu zaobserwowana została dla supernatantu pochodzącego z AJD2 pAD CUT_FS oraz AJD 2 pAD Lip2_CUT_FS po 48 h hodowli. Co ciekawe, po 72 h hodowli stężenie ε-PCL zmierzonego w płynie pohodowlanym była znacznie mniejsza, co może sugerować jego dalszą konwersję przez obecne w supernatancie natywne enzymy. Analiza

chromatograficzna produktu rozpadu PCL podczas hodowli wskazała również, że szczep kontrolny AJD 2 w bardzo małym stopniu degraduje ten polimer.

Wyniki uzyskane w Publikacji 2 wskazały jednoznacznie, że największy potencjał degradacyjny wykazywany jest przez szczepy AJD 2 pAD CUT_FS oraz AJD 2 pAD Lip2_CUT_FS. W oparciu o te dane przeprowadzono badania rozkładu poli(tereftalanu etylenu) (PET) przez wskazane szczepy.

4.3. **Publikacja 3- Kosiorowska, K.E., Biniarz, P., Dobrowolski, A., Leluk, K., Mirończuk, A.M., 2022. Metabolic engineering of *Yarrowia lipolytica* for poly(ethylene terephthalate) degradation. Sci. Total Environ. 831, 154841**.

Publikacja 3 opisuje wykorzystanie zmodyfikowanego szczepu drożdży *Y. lipolytica*,posiadającego zdolność do zewnątrzkomórkowej produkcji kutynazy z *F. solani* (AJD 2 pAD CUT_FS), uzyskanego w poprzedniej pracy (P2), do rozkładu poli(tereftalanu etylenu) (PET). Produktami degradacji PET są tereftalan bis(2-hydroksyetylu) (BHET), kwas mono(2-hydroksyetylo)tereftalowy (MHET), kwas tereftalowy (TPA) oraz glikol etylenowy (EG) (P3; Rys. 1.) W badaniach wykorzystano analizę ULPC do sprawdzania procesu hydrolizy PET.

W pierwszej fazie badań, zdolność do degradacji PET sprawdzana była na małej skali laboratoryjnej w płytkach głębokodołkowych, podczas której zauważono stały dobowy przyrost ilości TPA, natomiast ilości MHET zmierzonego w pozyskanych próbach były zmienne, co jest związane ze zdolnością do hydrolizy tego związku przez enzym kutynazy (P3; Rys. 2). Hodowle w małej skali laboratoryjnej wykorzystane zostały również do sprawdzenia wpływu suplementów w różnych stężeniach, takich jak sole nieorganiczne, oliwa z oliwek i olej rzepakowy na poziom degradacji PET. Wyniki przedstawione w materiałach dodatkowych do Publikacji 3 wskazały, że jedynie dodatek chlorku wapnia ($CaCl_2$) w końcowych stężeniu 2,5 mM miał istotny wpływ na zwiększenie ilości uwolnionego TPA przy jednoczesnym zmniejszeniu ilości uwalnianego MHET, co w porównaniu do hodowli kontrolnych prowadzonych bez suplementacji daje podobny sumaryczny wynik ilości uwolnionych produktów degradacji.

Hodowle przeprowadzone w płytkach głębokodołkowych potwierdziły zdolność szczepu AJD 2 pAD CUT_FS do hydrolizy PET na TPA oraz MHET. BHET oraz EG nie

zostały wykryte podczas analizy chromatograficznej, a późniejsze badania uzupełniające wykazały, że ma to związek ze zdolnością zmodyfikowanych drożdży *Y. lipolytica* do hydrolizy BHET do MHET oraz TPA i asymilacji EG podczas hodowli (P4). W oparciu o wyniki uzyskane w hodowlach w małej skali laboratoryjnej, przeniesiono doświadczenie do większej skali laboratoryjnej z użyciem 0,3 L kolb Erlenmeyera oraz zwiększono długość kultywacji drożdży. Wyniki analizy supernatantów pod względem obecnego w nich stężenia produktów degradacji PET wskazały, że zarówno ilości TPA, jak i MHET zwiększają się wraz z czasem trwania hodowli, a największy dzienny przyrost uwolnionych analitów w obu przypadkach następuje między 216 a 240 h kultywacji. Zgodnie z założeniami, wartości zmierzonych stężeń w hodowlach prowadzonych w kolbach są większe od tych uzyskanych w małej skali laboratoryjnej (P3; Rys. 3).

Degradacja PET prowadzona z użyciem enzymów zdolnych do hydrolizy wiązań estrowych obecnych w tym poliestrze wymaga zapewnienia środowiska umożliwiającego przeprowadzenie ataku nukleofilowego przez serynę obecną w centrum aktywnym kutynazy na karbonylowy węgiel obecny w łańcuchu polimeru. Ze względu na ten fakt, w próbkach pobieranych z hodowli prowadzonych w płytkach głębokodołkowych oraz kolbach Erlenmeyera, każda próbka została skontrolowana pod względem pH, celem ustalenia trendu, jaki występuje podczas kultywacji drożdży *Y. lipolytica* w zastosowanym podłożu. Wyniki przedstawione w materiałach dodatkowych do Publikacji 3 (P3; Sup. Rys. S2) wskazują, że podczas hodowli drożdży w małej skali alkalizacja podłoża następuje wolniej niż w przypadku hodowli prowadzonych w kolbach, jednakże, warunki umożliwiające zajście wydajnej hydrolizy zostają zapewnione bez konieczności zastosowania podłoża buforowanego.

Kolejnym etapem badań było przeniesienie degradacji PET bezpośrednio w hodowli drożdży do dużej skali laboratoryjnej, którą wykonaliśmy z użyciem bioreaktora o objętości roboczej 1 L. Wykorzystanie reaktora laboratoryjnego pozwoliło na pełną kontrolę procesu związaną z utrzymywaniem stałego pH hodowli na poziomie 8,5, co odpowiada warunkom optymalnym dla działania kutynazy. Zgodnie z wynikami uzyskanymi w hodowlach bioreaktorowych, próba zwiększenia skali procesu zakończyła się powodzeniem. Stężenie TPA zmierzone po 240 h przekroczyła 1,5 gL^{-1}, natomiast ilość MHET wahała się w czasie hodowli a największą ilość zmierzono po 216 h hodowli i wynosiła ona 0,45 gL^{-1} (P3; Rys. 4).

Równolegle do badań z użyciem szczepu AJD 2 pAD CUT_FS prowadzono hodowle kontrolne ze szczepem AJD 2, przy zastosowaniu takich samych warunków kultywacji. W żadnej z prób kontrolnych pobranych z hodowli prowadzonych w różnych skalach laboratoryjnych nie stwierdzono obecności produktów degradacji PET. Wynik ten wskazuje, że dzikie szczepy *Y. lipolytica* nie mają zdolności do dekompozycji tego tworzywa sztucznego w danych warunkach.

Przeanalizowanie danych uzyskanych z hodowli w trzech różnych skalach laboratoryjnych ukazało interesujący fenomen zmiany stosunku ilości uwolnionych produktów degradacji (MHET oraz TPA) zależnych od zastosowanej objętości roboczej. Zaobserwowano, że stosunek TPA do MHET (TPA: MHET) mierzony w ostatnim dniu hodowli w płytkach głębokodołkowych wynosił 0,55:1, dla hodowli w kolbach 2,95:1, a dla hodowli w bioreaktorach 4,63:1. Fakt ten sugeruje, że oprócz korzyści wynikających ze zwiększonej efektywności procesu prowadzonego z użyciem reaktorów laboratoryjnych, w eksperymentach prowadzonych z ich użyciem powstawanie terminalnego produktu rozpadu PET (TPA) jest faworyzowane.

W badaniach przeprowadzonych w ramach Publikacji 3 zdeterminowano również zdolność degradacji folii PET podczas długotrwałej hodowli ze szczepem AJD 2 pAD CUT_FS. Podczas tej fazy pracy, próbki folii pobrane po miesięcznej hodowli ze szczepem kontrolnym (AJD 2) oraz mutantem zostały poddane analizie z użyciem skaningowego mikroskopu elektronowego (SEM). Folia PET inkubowana w kulturze kontrolnej (P3;Rys. 5A) nie wykazuje znaczących uszkodzeń powierzchni. Widoczne są na niej niewielkie zarysowania, które prawdopodobnie powstały podczas procesów produkcji materiału lub przygotowywania folii do eksperymentu. Wynik ten świadczy, że szczep kontrolny nie wpływa na uszkodzenie polimeru. Próbki folii pobrane z hodowli AJD 2 pAD CUT_FS wykazują widoczne zmiany w strukturze powierzchni tworzywa. Na powierzchni tworzywa stwierdzono liczne uszkodzenia, nierówności, pęknięcia i ubytki.

Badania nad zdolnością degradacji PET przez szczep AJD 2 CUT_FS wskazały na możliwość zastosowania go do bezpośredniej hydrolizy tego polimeru podczas fermentacji prowadzonej w 28 °C bez konieczności wcześniejszej obróbki tworzywa sztucznego. Dodatkowo, połączenie naturalnej zdolności *Y. lipolytica* do utylizacji atypowych źródeł węgla oraz ekspresji heterologicznej kutynazy zaowocowało degradacją PET niewymagającej dużego nakładu kosztów.

4.4. **<u>Publikacja 4-</u> 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.**

Publikacja 4 przedstawia badania związane z inżynierią genetyczną drożdży *Yarrowia lipolytica* mającą na celu stworzenie szczepu zdolnego do zewnątrzkomórkowej produkcji enzymu PETazy z bakterii *Ideonella sakaiensis* (6EQD_A) AJD 2 pAD PET_IS oraz optymalizację procesu degradacji PET. Pierwszym etapem pracy było sprawdzenie funkcjonalności wklonowanego genu z użyciem metody qRT-PCR oraz porównanie profilu wzrostu utworzonego szczepu ze szczepem kontrolnym (P4; Rys. 1). Następnie zweryfikowano zdolność szczepów drożdży wykorzystanych w pracy (AJD 2 oraz AJD 2 pAD PET_IS) do wzrostu na produktach rozpadu PET, takich jak MHET, TPA oraz EG. Uzyskane wyniki wskazują, że zarówno szczep kontrolny jak i szczep zmodyfikowany nie utylizują TPA (P4; Rys. 2A). Zaobserwowano niewielki wzrost stężenia tego związku w pożywce, jednak biorąc pod uwagę czas trwania hodowli (168 h), wzrost ten może być związany z procesem parowania. Eksperyment mający na celu sprawdzenie zdolności degradacji MHET wykazał, że szczep AJD 2 pAD PET_IS całkowicie hydrolizował ten związek w czasie 96 godzin hodowli, pojawiał się produkt jego rozkładu TPA. Natomiast w hodowli kontrolnej, AJD 2, nie zauważono spadku stężenia MHET(P4; Rys. 2B). Wyniki wskazują, że PETaza z *I. sakaiensis* produkowana przez zmodyfikowany szczep *Y. lipolytica* jest zdolna do efektywnej hydrolizy MHET bez konieczności obecności dodatkowych enzymów z rodziny hydrolaz. Badanie zdolności wzrostu drożdży w podłożu z dodatkiem glikolu etylenowego wykazało, że zarówno szczep kontrolny AJD 2, jak i AJD 2 pAD PET_IS mają zdolność do asymilacji tego związku (P4; Rys. 2C). Przeprowadzone badania miały kluczowe znaczenie dla sprawdzenia, czy zastosowana metoda analizy ilości produktów rozpadów nie jest zafałszowana przez pobór badanego analitu obecnego w supernatancie hodowli.

Po wykluczeniu asymilacji TPA przez szczepy AJD 2 oraz AJD 2 pAD PET_IS, wykonano badania degradacji proszku PET bezpośrednio w hodowli drożdży *Y. lipolytica.* Równolegle badano wpływ dodatków na poziom degradacji PET, takich jak sole nieorganiczne i oliwa z oliwek w różnych stężeniach. Stopień degradacji oszacowany został na podstawie ilości uwolnionego TPA podczas hydrolizy tworzywa sztucznego. Zgodnie z wynikami uzyskanymi we wcześniejszym etapie badań, gdy zaobserwowano hydrolizę MHET do TPA przez enzym PETazy oraz asymilację EG, związki te nie zostały wykryte

w płynie pohodowlanym. Ponadto w żadnej z badanych próbek nie zaobserwowano produktu BHET, którego zdolność hydrolizy przez *Y. lipolytica* potwierdziliśmy już we wstępnych badaniach wykonanych na początku pracy w temacie degradacji PET przez te drożdże. Badanie przesiewowe potencjalnych aktywatorów hydrolizy PET, zostało wykonane w małej skali laboratoryjnej z użyciem płytek głębokodołkowych. Uzyskane wyniki, wskazały, że zwiększenie ilości uwolnionego TPA podczas degradacji PET bezpośrednio w hodowli następuje w przypadku kultywacji suplementowanych $MnSO_4 \cdot 7H2O$, przy końcowym stężeniu soli wynoszącym 1 mM oraz 2,5 mM, gdy zmierzona ilość TPA wynosiła odpowiednio 30 mgL^{-1} i 75 mgL^{-1}. Istotny wpływ na hydrolizę PET miał również dodatek 1 mM $CuSO_4 \cdot 5H2O$, w którym stężenie TPA wynosiło 62 mgL^{-1}. Oprócz wymienionych soli, pozytywny efekt na wydajność hydrolizy PET miał również dodatek oliwy z oliwek w końcowym stężeniu 1 % (P4; Rys. 3). Co ciekawe, zwiększona ilość tego suplementu (2 %) nie wpłynęła pozytywnie na proces hydrolizy. Prawdopodobnie zaobserwowana różnica ma związek ze zdolnością drożdży *Y. lipolytica* do produkcji biosurfaktantów, która stymulowana jest już przy niskim stężeniu oliwy z oliwek (1 %), natomiast w wyższym (2 %) następuje ich przyleganie do ściany komórkowej. Takie stężenie oliwy z oliwek może również aktywować natywne lipazy, które ,działając synergicznie z PETazą, mogą zwiększać poziom degradacji PET (Carniel et al., 2017; Papanikolaou et al., 2007).

Po przeprowadzeniu badań pilotażowych w celu wybrania potencjalnych suplementów, które mogłyby zwiększyć degradację PET w hodowli drożdży, podjęliśmy próbę zwiększenia skali procesu. W tym celu wykonane zostały hodowle z wykorzystaniem 0,3 L kolb Erlenmeyera. Przeniesienie procesu degradacji PET w hodowlach suplementowanych na większą skalę wykazało, że istotny wpływ soli obserwowany w małej skali laboratoryjnej, w tym przypadku jest niwelowany. Wynik ten może być związany z warunkami napowietrzania i szybkością wzrostu komórek. W porównaniu z małą skalą, komórki drożdży kultywowane w kolbach miały zapewnione lepsze warunki do wzrostu, a dodatek soli w tych warunkach miał efekt odwrotny do wcześniej obserwowanego. W przeciwieństwie do braku pozytywnego wpływu soli na degradację PET w hodowlach przeprowadzonych w kolbach, dodatek oliwy z oliwek w końcowym stężeniu wynoszącym 1%, determinował statystycznie istotny wpływ na wzrost poziomu degradacji tego polimeru o 66 %. Podobnie jak w przypadku Publikacji 3, badania supernatantów pozyskanych z kultywacji szczepu kontrolnego AJD 2 w warunkach identycznych jak mutanta, nie wykazały obecności produktów degradacji, co potwierdza brak zdolności tego szczepu do degradacji PET.

Ostatnim etapem badań wykonanych w ramach pracy przedstawionej w Publikacji 4 była analiza powierzchni folii PET pozyskanych z miesięcznych hodowli z użyciem szczepów AJD 2 oraz AJD 2 pAD PET_IS w podłożach z i bez suplementacji oliwą z oliwek. Struktura folii pozyskanych z hodowli była badana z użyciem skaningowego mikroskopu elektronowego (SEM). W przypadku użycia szczepu AJD 2, podobnie jak w Publikacji 3, nie zaobserwowano istotnych zmian na powierzchni tworzywa sztucznego. Dla hodowli szczepu AJD 2 pAD PET_IS w hodowli bez dodatku oliwy z oliwek zaobserwowano natomiast widoczne, rozległe uszkodzenia powierzchni materiału (P4, Rys. 5). Co ciekawe, w przypadku zastosowania podłoża suplementowanego, folie pozyskane z hodowli mutanta, oprócz widocznych wyżłobień zawierają także kratery o nieregularnym kształcie.

Zaprezentowane w ramach niniejszych badań wyniki wskazują na możliwość zastosowania drożdży *Y. lipolytica* jak dobrego kandydata do zewnątrzkomórkowej produkcji PETazy z *I. sakaiensis*, a wydajna hydroliza PET może zostać przeprowadzona bezpośrednio w hodowli zmodyfikowanych drożdży. Przeprowadzone badania wykazały również, że spośród wszystkich przetestowanych potencjalnych suplementów hodowlanych, jedynie dodatek określonego stężenia oliwy z oliwek do podłoża hodowlanego drożdży może istotnie wpływać na wzrost ilości uwolnionego TPA. Widoczne zmiany powierzchni plastiku powstałe po hodowli z wygenerowanym mutantem wskazują również na brak konieczności wstępnej obróbki plastiku w celu efektywnej degradacji.

5. Podsumowanie

Niniejsza rozprawa doktorska porusza istotny temat związany z zanieczyszczeniem środowiska odpadami tworzyw sztucznych, których nagromadzenie negatywnie wpływa na wiele ekosystemów. Przedstawione propozycje rozwiązania problemu, związane są z zastosowaniem metody recyklingu biologicznego z wykorzystaniem *Yarrowia lipolytica*. Zastosowanie tych niekonwencjonalnych drożdży jako organizmu gospodarza do zewnątrzkomórkowej produkcji enzymów z klasy hydrolaz zdolnych do hydrolizy wiązań estrowych obecnych w materiałach plastikowych, wprowadza nowe możliwości w zakresie tematyki degradacji tworzyw sztucznych.

Badania wykonane w ramach niniejszej pracy wskazują, że spośród wygenerowanych szczepów drożdży *Y. lipolytica*, najbardziej obiecującymi są szczepy produkujące zewnątrzkomórkowo kutynazę z *F. solani* oraz PETazę z *I. sakaiensis*. Przeprowadzone eksperymenty wykazały, że szczep AJD 2 pAD CUT_FS może wydajnie hydrolizować zarówno wiązania estrowe obecne w poliestrach alifatycznych (PCL) jak i aromatycznych (PET). Szczep AJD pAD PET_IS natomiast, skutecznie hydrolizuje wiązania obecne w PET, a dodatkowa suplementacja hodowli oliwą z oliwek może istotnie zwiększać wydajność tego procesu. Analiza SEM folii PET wskazała ponadto na istotną różnicę w funkcjonowaniu zastosowanych enzymów. Przy użyciu kutynazy zaobserwowano powierzchniowe działanie enzymu, co ma związek ze zwiększeniem hydrofilowości polimeru przez to białko, natomiast w przypadku PETazy odnotowano znaczną erozję powierzchni tworzywa spowodowaną działaniem enzymu również w wewnętrznej warstwie polimeru.

Zastosowane w pracy zmodyfikowane genetycznie drożdże *Y. lipolytica* rozszerzają perspektywę wykorzystania zaprezentowanej metody w kolejnych badaniach optymalizujących proces rozkładu poliestrów. Możliwość przeprowadzenia procesu degradacji tworzyw sztucznych w niskiej temperaturze (28 °C), w stosunkowo krótkim czasie oraz brak konieczności wstępnej obróbki materiału plastikowego, wyróżniają zaproponowany proces spośród opublikowanych dotychczas doniesień w tym temacie. Dodatkowo badania wykazały możliwość zwiększenia skali procesu niepowodującej obniżenia efektywności degradacji, a badania przesiewowe suplementacji hodowli solami nieorganicznymi zdeterminowała brak konieczności urozmaicania podłoża hodowlanego dodatkowymi komponentami.

6. Wnioski

Badania wykonane w ramach niniejszej pracy związane były z badaniem zdolności zmodyfikowanych drożdży *Yarrowia lipolytica* do rozkładu tworzyw sztucznych. Materiałami modelowymi, z wykorzystaniem których podjęto próbę określenia potencjału wygenerowanych szczepów do dekompozycji plastiku był: poli(ε-kaprolakton) (PCL) oraz poli(tereftalan etylenu) (PET). Po przeanalizowaniu uzyskanych danych, najważniejsze wnioski są następujące:

1. Skonstruowane szczepy drożdży *Y. lipolytica* funkcjonalnie ekspresjonują wklonowane geny kutynaz grzybowych oraz bakteryjnej PETazy.
2. Szczepy AJD 2 pAD Lip2, AJD 2 pAD CUT_FS, AJD 2 pAD Lip2_CUT_FS, AJD 2 pAD CUT_TR oraz AJD Lip2_CUT_TR były zdolne do hydrolizowania wiązań estrowych obecnych w poliestrach alifatycznych (PCL).
3. Spośród dwóch przebadanych kutynaz grzybowych, lepszy potencjał w badaniach degradacyjnych w testowanych warunkach wykazuje kutynaza z *Fusarium solani*.
4. Badania z wykorzystaniem PET wykazały, że szczepy AJD 2 pAD CUT_FS oraz AJD 2 pAD PET_IS posiadają zdolność do hydrolizy wiązań obecnych w tym poliestrze aromatycznym bezpośrednio w hodowli mikrobiologicznej.
5. Szczep kontrolny AJD 2 nie posiada zdolności degradacji PET.
6. Użyte w pracy niekonwencjonalne drożdże *Y. lipolytica* posiadają zdolność do asymilacji glikolu etylenowego (EG), który jest jednym z terminalnych produktów rozpadu PET.
7. Żaden z przebadanych szczepów *Y. lipolytica* wykorzystany w badaniach nie asymilował TPA, natomiast hydroliza MHET zachodziła szybciej z wykorzystaniem szczepu AJD 2 pAD PET_IS.
8. Uzyskane wyniki wskazują, że wraz ze zwiększeniem objętości roboczej hodowli, stosunek ilości TPA do MHET zmienia się na korzyść terminalnego produktu rozpadu (TPA).
9. Istotny wpływ na zwiększenie wydajności degradacji przez AJD 2 pAD PET_IS został zaobserwowany dla dodatku do podłoża hodowlanego oliwy z oliwek w końcowym stężeniu 1 %.
10. Analiza struktury folii PET po długotrwałej hodowli z mutantami wykazała, że zarówno szczep AJD 2 pAD CUT_FS jak i AJD w pAD PET_IS powodują liczne

uszkodzenia powierzchni folii PET, która różni się rodzajem uszkodzeń w zależności od zastosowanego szczepu i suplementacji podłoża.

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8. Dorobek naukowy

Publikacje

1. **Kosiorowska, K.E.**, Moreno, A.D., Iglesias, R., Leluk, K., Mironczuk, A.M., 2022. Production of Petase by Engineered *Yarrowia Lipolytica* for Efficient Poly(Ethylene Terephthalate) Biodegradation. Sci. Total Environ. 846, 157358.
   (IF: 10,753; MEiN: 200)
2. **Kosiorowska, K.E.**, Biniarz, P., Dobrowolski, A., Leluk, K., Mirończuk, A.M., 2022. Metabolic engineering of *Yarrowia lipolytica* for poly(ethylene terephthalate) degradation. Sci. Total Environ. 831, 154841.
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Doniesienia konferencyjne

1. **Katarzyna E. Kosiorowska**, Aleksandra M. Mirończuk. Enhanced poly(ethylene terephthalate) degradation by the modified *Yarrowia lipolytica* strains. The Biochemistry Global Summit (25th IUBMB Congress, the 46th FEBS Congress and the 15th PABMB Congress) 9.07.2022-14.07.2022. Lizbona Portugalia
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2. A.M. Mironczuk, I, A. Urbanek, **K.E. Kosiorowska**. Biodegradation of the polyesters by the engineered yeast *Yarrowia lipolytica*. The Biochemistry Global Summit (25th IUBMB Congress, the 46th FEBS Congress and the 15th PABMB Congress) 9.07.2022-14.07.2022. Lizbona Portugalia
   (Poster)
3. **Katarzyna E. Kosiorowska**, Aleksandra Maria Mirończuk. Genetic engineering of *Yarrowia lipolytica* yeast for poly(ethylene terephthalate) degradation. 1st Polish Yeast Conference. 22.06.2022-24.06.2022. Rzeszów, Polska
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5. **Katarzyna E. Kosiorowska**, Xymena Połomska, Guokun Wang, Irina Borodina, Aleksandra M. Mirończuk. Biodegradation of aliphatic poliester by genetically engineered strains of the *Yarrowia lipolytica* yeast. 15th International Congress on Yeasts (ICY) and the 30th International Conference on Yeast Genetics and Molecular Biologs (ICYGMB30). 24.08.2022-27.08.2022. Wiedeń, Austria (online)
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6. **Katarzyna E. Kosiorowska**, Irina Borodina, Guokun Wang, Aleksandra M. Mirończuk. Metabolic Engineering Of *Yarrowia lipolytica* For Plastic Degradation. XXIX International Conference on Yeast Genetics and Molecular Biology; 17-08-2019-22-08-2019 Göteborg, Szwecja
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7. **Katarzyna E. Kosiorowska**, Irina Borodina, Guokun Wang, Aleksandra M. Mirończuk. Inżynieria metaboliczna drożdży *Yarrowia lipolytica* do degradacji tworzyw sztucznych. 31.11.2019-1.12.2019. Wrocław, Polska
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8. **Katarzyna E. Kosiorowska**. Plastik na świecie- kiedyś, dziś i w przyszłości. Plastikowa Planeta. 31.11.2019-1.12.2019. Wrocław, Polska
   (Wystąpienie ustne)

Staże i szkolenia

1. 7.09.2022 – 9.09.2022

   Szkolenie Yeast4Bio

   Temat: *Metabolite profiling of non-conventional yeasts, including lipids (carotenoids) by U/HPLC-PDA and GC-MS*

   Royal Holloway University of London, Imperial College London, Londyn, Wielka Brytania

   Finansowanie: COST

2. 05.11.2021 – 05.12.2021

   Staż naukowy w CIEMAT - THE CENTER FOR ENERGY, ENVIRONMENTAL AND TECHNOLOGICAL RESEARCH, Madryt, Hiszpania

   Temat: *Pre-treatment of plastic material to enhance its degradation by modified Yarrowia lipolytica strains.*

   Opiekun naukowy: Antonio David Moreno Garcia

   Finansowanie: projekt PROM, Narodowa Agencja Wymiany Akademickiej (NAWA)

3. 20.09.2021 – 30.10.2021

   Staż naukowy w CIEMAT - THE CENTER FOR ENERGY, ENVIRONMENTAL AND TECHNOLOGICAL RESEARCH, Madryt, Hiszpania

   Temat: *Optimization of culture condition for efficient degradation of plastic material.*

   Opiekun naukowy: Antonio David Moreno Garcia

   Finansowanie: projekt INCREaSE, NAWA

4. 1.02.2019 – 10.05.2019

   Staż naukowy w DTU - TECHNICAL UNIVERSITY OF DENMARK, Kgs. Lyngby, Dania

   Temat: *Yeast metabolic engineering*

   Opiekun naukowy: Irina Borodina

   Finansowanie: Erasmus +

Granty i Projekty badawcze

1. *Pre-treatment of plastic material to enhance its degradation by modified Yarrowia lipolytica strains*. Projekt PROM, Narodowa Agencja Wymiany Akademickiej nr PPI/PRO/2019/1/0004/U/001. Staż zagraniczny realizowany w CIEMAT (Madryt, Hiszpania) w terminie 05.11.2021 – 05.12.2021
2. *Optimization of culture condition for efficient degradation of plastic material*. Projekt INCREaSE, Narodowa Agencja Wymiany Akademickiej. Staż zagraniczny realizowany w CIEMAT (Madryt, Hiszpania) w terminie 20.09.2021 – 30.10.2021
3. *Badanie wpływu alternatywnych źródeł węgla na proces biosyntezy lipidów w drożdżach Yarrowia lipolytica*, Narodowe Centrum Nauki, SONATA BIS 7 nr UMO-2017/26/E/NZ9/00975, stypendysta.
4. *Badanie zdolności fizjologicznych drożdży Yarrowia lipolytica do rozkładu tworzyw sztucznych*, Narodowe Centrum Nauki, OPUS 14 nr UMO-2017/27/B/NZ9/02218, wykonawca.

9. Oświadczenia współautorów publikacji

Załącznik nr 4

Aleksandra M. Mirończuk
imię i nazwisko

Wrocław, 25.07.2022
(miejscowość i data)

Uniwersytet Przyrodniczy we Wrocławiu, Wydział Biotechnologii i Nauk o Żywności
Chełmońskiego 37, 51-630, Wrocław, Polska
Afiliacja

OŚWIADCZENIE

Oświadczam, że w pracach:

Katarzyna E. Kosiorowska, Xymena Połomska, Guokun Wang, Irina Borodina, **Aleksandra M. Mirończuk**. Efficient biodegradation of aliphatic polyester by genetically engineered strains of the yeast *Yarrowia lipolytica*. International Biodeterioration & Biodegradation, Volume 161, July 2021, 105232, https://doi.org/10.1016/j.ibiod.2021.105232

Urbanek, A.K., Kosiorowska, K.E., **Mirończuk, A.M.**, 2021. Current Knowledge on Polyethylene Terephthalate Degradation by Genetically Modified Microorganisms 9, 1–15. https://doi.org/10.3389/fbioe.2021.771133

Kosiorowska, K.E., Biniarz, P., Dobrowolski, A., Leluk, K., **Mirończuk, A.M.**, 2022. Metabolic engineering of *Yarrowia lipolytica* for poly(ethylene terephthalate) degradation. Sci. Total Environ. 831, 154841. https://doi.org/10.1016/j.scitotenv.2022.154841

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Poly(Ethylene Terephthalate) Biodegradation. Sci. Total Environ. 846, 157358. https://doi.org/https://doi.org/10.1016/j.scitotenv.2022.157358

mój udział polegał na ustaleniu koncepcji oraz planu badań, krytycznej weryfikacji manuskryptu pod kątem istotnych treści intelektualnych oraz ostatecznym zatwierdzeniu wersji do publikacji.

Wkład Katarzyny E. Kosiorowskiej w pracy nad przygotowaniem powyższych manuskryptów wiązał się z przeprowadzeniem badań, analizą wyników, wizualizacją danych, analizą dotychczasowych doniesień naukowych i ich dyskusją a także w napisaniu manuskryptów.

25.07.2022

data i podpis

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Wrocław, 18.05.2022r.

(miejscowość i data)

OŚWIADCZENIE

Oświadczam, że w pracy:

> **Urbanek, A.K**., Kosiorowska, K.E., Mirończuk, A.M., 2021. Current Knowledge on Polyethylene Terephthalate Degradation by Genetically Modified Microorganisms 9, 1–15. https://doi.org/10.3389/fbioe.2021.771133

mój udział polegał przygotowaniu części manuskryptu związanej z genetycznie zmodyfikowanymi mikroorganizmami posiadającymi zdolność do degradacji poli(tereftalanu etylenu) oraz przygotowaniu tabel oraz figur.

Katarzyna E. Kosiorowska w przygotowaniu niniejszej publikacji odpowiadała za analizę doniesień naukowych związanych z modyfikacją enzymów zdolnych do degradacji poli(tereftalanu etylenu) takich jak PETaza, MHETaza, kutynazy oraz lipazy a także odpowiadała za napisanie manuskryptu.

Aneta Urbanek

18.05.2022r.

datai podpis

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Wrocław 16.05.2022
(miejscowość i data)

Afiliacja

OŚWIADCZENIE

Oświadczam, że w pracy:

Katarzyna E. Kosiorowska, **Xymena Połomska**, Guokun Wang, Irina Borodina, Aleksandra M. Mirończuk. Efficient biodegradation of aliphatic polyester by genetically engineered strains of the yeast *Yarrowia lipolytica*. International Biodeterioration & Biodegradation, Volume 161, July 2021, 105232, https://doi.org/10.1016/j.ibiod.2021.105232

mój udział polegał na przeprowadzeniu analizy filogenetycznej enzymów z klasy hydrolaz oraz interpretacji uzyskanych wyników.

Wkład Katarzyny E. Kosiorowskiej polegał na przeprowadzeniu badań laboratoryjnych, analizie wyników, przygotowaniu figur oraz wersji roboczej manuskryptu.

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my contribution was revising the manuscript critically for important intellectual content. Contribution of Katarzyna E. Kosiorowska in the preparation of the above-mentioned manuscript consisted of performing the laboratory investigations, analysing the obtained results, preparing the figures and writing the manuscript.

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my contribution was revising the manuscript critically for important intellectual content. Contribution of Katarzyna E. Kosiorowska in the preparation of the above-mentioned manuscript consisted of performing the laboratory investigations, analysing the obtained results, preparing the figures and writing the manuscript.

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mój udział polegał na opracowaniu metodologii do analizy powstających produktów degradacji z wykorzystaniem wysokosprawnej chromatografii cieczowej oraz analizie uzyskanych wyników.
Katarzyna E. Kosiorowska przygotowując powyższą publikację odpowiadała za przeprowadzenie badań laboratoryjnych, opracowanie wyników, przygotowanie figur oraz napisaniu manuskryptu .

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Kosiorowska, K.E., Biniarz, P., **Dobrowolski, A**., Leluk, K., Mirończuk, A.M., 2022. Metabolic engineering of *Yarrowia lipolytica* for poly(ethylene terephthalate) degradation. Sci. Total Environ. 831, 154841.

https://doi.org/10.1016/j.scitotenv.2022.154841

mój udział polegał na przygotowaniu części szczepów drożdżowych oraz redagowaniu treści manuskryptu. Katarzyna E. Kosiorowska przygotowując powyższą odpowiadała za przeprowadzenie badań laboratoryjnych związanych z degradacją poli(tereftalanu etylenu), interpretacji uzyskanych wyników, przygotowaniu figur umieszczonych w publikacji oraz napisaniu manuskryptu.

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Data i podpis

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degradation. Sci. Total Environ. 831, 154841. https ://doi.org/10.1016/j.scitotenv.2022.154841

Kosiorowska, K.E., Moreno, A.D., Iglesias, R., **Leluk, K**., Mironczuk, A.M., 2022. Production of PETase by Engineered *Yarrowia Lipolytica* for Efficient Poly(Ethylene Terephthalate) Biodegradation. Sci. Total Environ. 846, 157358. https://doi.org/https://doi.org 10.1016/j.scitotenv.2022.157358

mój wkład polegał na analizie fragmentów folii tereftalanu polietylenu z wykorzystaniem skaningowej mikroskopii elektronowej oraz redagowaniu treści manuskryptów.

Katarzyna E. Kosiorowska przygotowując powyższe publikacje odpowiadała za przeprowadzenie badań, analizę wyników, wizualizację danych oraz napisaniu manuskryptów.

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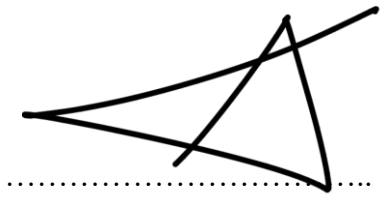

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REVIEW
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Current Knowledge on Polyethylene Terephthalate Degradation by Genetically Modified Microorganisms

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The global production of polyethylene terephthalate (PET) is estimated to reach 87.16 million metric tons by 2022. After a single use, a remarkable part of PET is accumulated in the natural environment as plastic waste. Due to high hydrophobicity and high molecular weight, PET is hardly biodegraded by wild-type microorganisms. To solve the global problem of uncontrolled pollution by PET, the degradation of plastic by genetically modified microorganisms has become a promising alternative for the plastic circular economy. In recent years many studies have been conducted to improve the microbial capacity for PET degradation. In this review, we summarize the current knowledge about metabolic engineering of microorganisms and protein engineering for increased biodegradation of PET. The focus is on mutations introduced to the enzymes of the hydrolase class—PETase, MHETase and cutinase—which in the last few years have attracted growing interest for the PET degradation processes. The modifications described in this work summarize the results obtained so far on the hydrolysis of polyethylene terephthalate based on the released degradation products of this polymer.

Keywords: plastic degradation, genetic engineering, microorganisms, PET, protein

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INTRODUCTION

Worldwide plastic production reached 348 million metric tons in 2017, and this number increases annually by ~5% (PlasticEurope, 2019; Brahney et al., 2020). Predictions about plastic waste accumulation in ecosystems suggest that in 2050 cumulative plastic waste production will reach over 25 billion tonnes, i.e., 3 times the current level (Geyer et al., 2017). The high resilience and persistence of plastic, previously considered an advantage, nowadays leads to the uncontrolled accumulation of waste in every ecosystem on the planet. Most plastics never completely disappear and only get fragmented into smaller pieces. The formed microplastics (1 μm—5 mm) and nanoplastics (<1 μm) spread all over the globe, reaching pristine regions separated from human activity. For instance, plastic particles have been found in the Arctic Polar Circle (Cózar et al., 2017), Antarctica (Waller et al., 2017), the high mountains (French Pyrenees) (Allen et al., 2019), the Mariana Trench (Gangadoo et al., 2020) and even in the rain in protected areas (Brahney et al., 2020). Easily transported microplastics are extremely dangerous to marine and seacoast animals. It is estimated that more than 800 animal species are affected by plastic waste, and around 90% of all seabirds ingest plastic (Wilcox et al., 2015). Both nanoplastics and microplastics were found in zooplankton and phytoplankton (Rummel et al., 2017), which are consumed by organisms from higher levels of the food chain. Hence microplastics are consumed and accumulated by invertebrates (Thompson et al., 2004). Moreover, it was shown that nanoplastics may reduce the survival of aquatic zooplankton and penetrate the blood-brain barrier in fish and cause behavioural disorders (Mattsson

et al., 2017). A recent study showed that crop plants are capable of effective uptake of microplastic and its transport from the roots to the shoots (Li et al., 2020). As it turns out, the ubiquitous plastics also affect the human body. The presence of microplastics was found in the lungs (Pauly et al., 1998) and faecal samples (Schwabl et al., 2019). *In vitro* studies have demonstrated the ability of microplastics to induce an immune response, oxidative stress, cytotoxicity, alteration of membrane integrity and variation in gene expression (Maeza et al., 2021).

Most of the produced plastic material has a fossil origin. Thermoplastic materials such as polyethylene (PE), polyurethane (PUR), polyvinyl chloride (PVC), polypropylene (PP), polystyrene (PS) and polyethylene terephthalate (PET) represent 80% of total global plastic usage (PlasticEurope, 2019). One of the most popular plastic materials used for packing (such as the production of bottles) is PET. PET is a polar, linear polymer of repeating units of aromatic terephthalic acid (TPA) and ethylene glycol (EG). The PET monomer is designated bis(2-hydroxyethyl) terephthalate (BHET). Owing to excellent mechanical and thermal properties, PET is mainly used for beverage bottles, foil, textile fibres and food containers (Danso et al., 2019; Taniguchi et al., 2019; Hiraga et al., 2020). The global production of PET reached 33 million metric tons in 2015 (Geyer et al., 2017) and is still increasing. The problem that has arisen with such enormous production of PET is partially solved by recycling. The main goal of recycling is to obtain new PET or recover the primary components such as TPA and EG so that they can be used as feedstock (Lange 2002). Nowadays, the recycling of PET is mainly based on chemical and mechanical methods. For instance, the mechanical recycling method for PET, melt extrusion, results in the production of rPET fibres from PET bottle waste (Park and Kim 2014), whereas the most common chemical method, glycolysis, degrades PET to BHET with a yield as high as 95% (Imran et al., 2013; Liu et al., 2020). Although these methods are commonly used, they still have some limitations such as spontaneous degradation during the lifetime of new PET obtained after re-extrusion (Park and Kim 2014) or requirement of high temperature (150–300°C) and catalysts in the glycolysis reaction. Especially using catalysts (metal-based, organic or ionic liquids) leads to the high cost of reagents and methodologies, a negative environmental impact and sometimes to the limitation to small-scale trials of reactions (Liu et al., 2020, Sang et al., 2020). In recent years, biological methods have been developed alongside the chemical and mechanical methods of PET recycling. Biological methods are promising and eco-friendly solutions for the decomposition of PET waste. Although PET is labelled as non-biodegradable, much research succeeded in the use of microorganisms or enzymes to break it down. A flagship example is the discovery of the bacterium *Ideonella sakaiensis* 201-F6 and the enzymes PETase and MHETase (Yoshida et al., 2016; Furukawa et al., 2019), which are the focus of many scientists due to very promising aspects of future management of PET. Other enzymes such as cutinases Thc_Cut1 and Thc_Cut2 from *Thermobifida cellulosilytica* DSM44535 (Acero et al., 2011), cutinase FsC from *Fusarium solani pisi* (Egmont and de Vlieg, 2000), cutinase HiC from *Humicola insolens* and lipase CALB from *Candida antarctica* (Carniel et al., 2017) or cutinase TfH from *Thermobifida fusca* DSM43793 (Müller et al., 2005) are also the subject of numerous studies. To date, scientists have verified 27 enzymes that degrade synthetic polymers (Danso et al., 2019), among which enzymes involved in the degradation of PET are typical serine hydrolases, e.g., cutinases (EC 3.1.1.74), lipases (EC 3.1.1.3), and carboxylesterases (EC 3.1.1.1) (Roth et al., 2014). Despite the knowledge of many enzymes, there are many unsolved issues regarding their practical use to degrade PET such as low thermal stability or transfer on an industrial scale (Sang et al., 2020). Much more investigation is needed for mutational developments of the enzyme's active site, which may help to overcome the limitations.

The degradability of the polymer depends on different factors such as shape, size, presence of various substituents, e.g., chloride atoms or benzene rings, and it decreases with the increase in the molecular weight (Kumari et al., 2019; Rose et al., 2020). The bottlenecks in plastic biodegradation are their high hydrophobicity, crystallinity, strong chemical bonds and high molecular weight (Urbanek et al., 2018). In the past years, a number of studies have been conducted in order to show that many microorganisms and enzymes are capable of degrading plastic. Researchers were mostly focused on the biodegradation performed by wild-type strains, isolated directly from different environments, especially from plastic contaminated areas.

Although this approach is justified due to the ubiquity of microorganisms and their diverse biodegradability, current studies should be more focused on improving these properties. Published reports show that naturally isolated microorganisms possess a limited capability for plastic degradation. Thus, more efficient production of enzymes and the improvement of enzymes' activity that would target specific materials with greater selectivity is a key to the improvement of the biodegradation rate of plastic. Employing metabolic engineering provides powerful opportunities in this field (**Figure 1**).

Here, we present the possibilities of genetic manipulation in order to obtain mutant enzymes with improved catalytic activity and thermostability in the hydrolysis of polyethylene terephthalate (PET) and other polymers.

Enzymes Involved in PET Degradation

Mostly, the biodegradation of PET is possible by the enzymatic activity of cutinases (EC 3.1.1.74) or PETase (EC 3.1.1.101) with the cooperation of MHETase (EC 2.1.1.102). A number of cutinases with PET biodegradable activity were found, e.g., cutinase from *Humicola insolens* (HiC), *Thermobifida fusca* (TfCut2), leaf-branch compost (LCC) (Tournier et al., 2020), and *Ideonella sakaiensis* (PETase and MHETase) (Yoshida et al., 2016; Furukawa et al., 2019). Cutinases are able to hydrolyse both ester bonds found in aliphatic and aromatic polyesters, hence their wide application in degradation studies of a broad range of plastic polymers (Sulaiman et al., 2012; Liu et al., 2019a). In contrast, PETase can hydrolyse ester bonds present only in aromatic polyesters (Austin et al., 2018). Enzymes involved in PET degradation belong to the esterase subclass and possess a catalytic triad characteristic for α/β-hydrolases (Ser-His-Asp). Ester bond hydrolysis is provided due to the nucleophilic

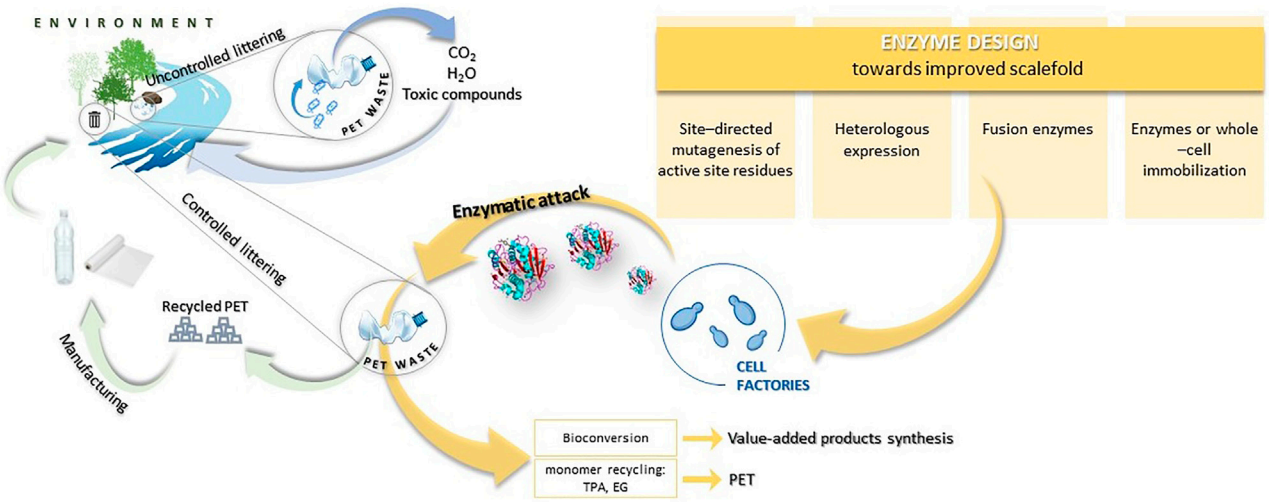

FIGURE 1 | Scheme of plastic circulation in the natural environment and the possibilities of the plastic circular economy.

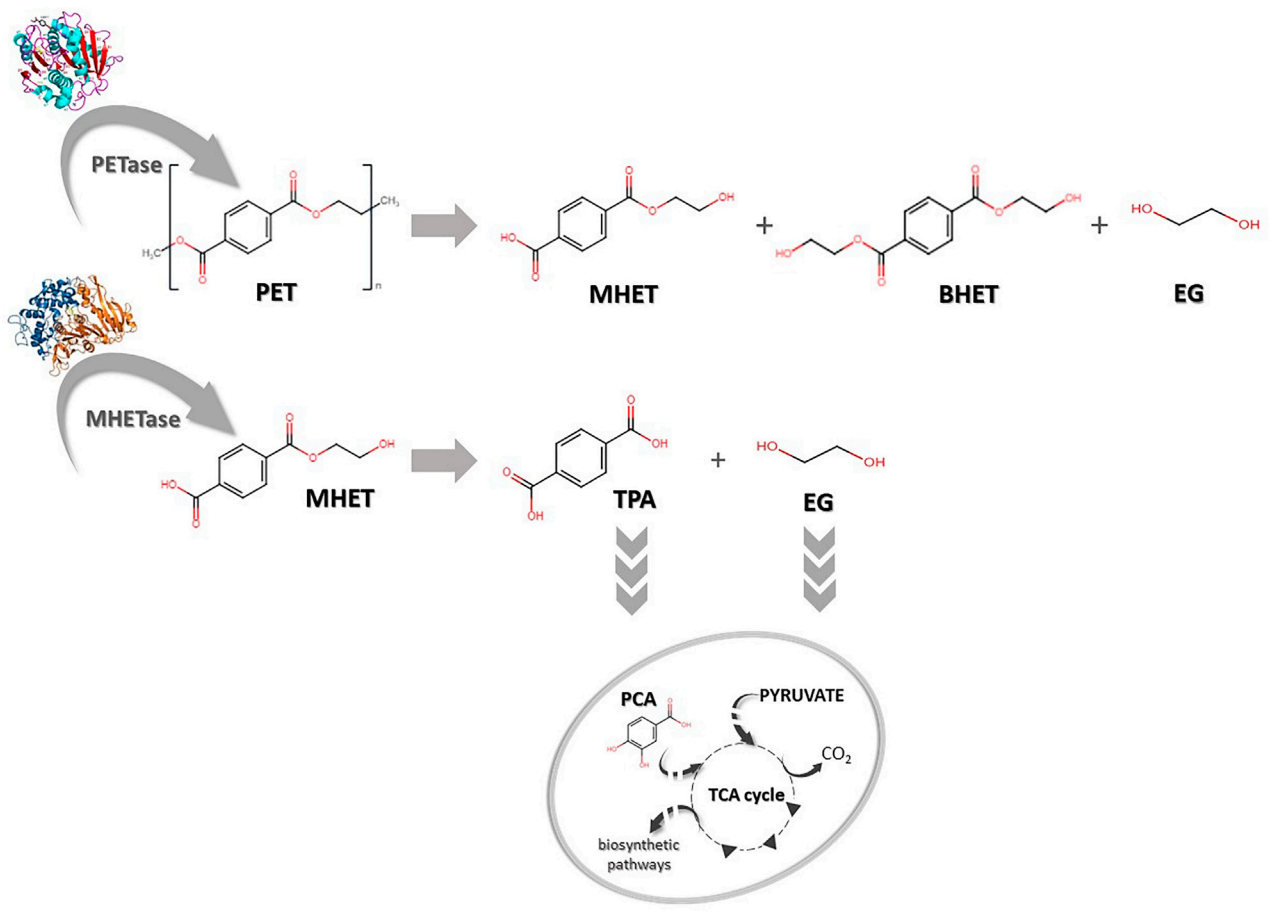

FIGURE 2 | Biodegradation of polyethylene terephthalate (PET). PET can be degraded into mono-(2-hydroxyethyl) terephthalate (MHET), bis-(2-hydroxyethyl) terephthalate (BHET) and ethylene glycol (EG) by PETase. MHET can later be hydrolysed to terephthalic acid (TPA) and EG by MHETase. TPA and EG can be further transported into the cell and converted to protocatechuic acid (PCA) and subsequently integrated into cellular metabolisms via the tricarboxylic acid cycle (TCA).

TABLE 1 | Genetic modifications of enzyme structure for enhancement of biodegradable abilities towards plastic.

Wild-type enzyme/strain	Wild-type microorganisms	Substrate specificity	Vector and host	Improvement in activity	Mutatation's information	References
PETase	*Ideonella sakaiensis*	PET	Plasmid: pET28a; Host: *E. coli* BL21 (DE3)	-R61A: 1.6 fold -L88F: 2.0 fold -I179F: 15.0 fold[a]	mutagenesis of six key residues around the substrate-binding groove in order to: create space around the active site; increase the hydrophobicity of the amino acids around the active site; improve the affinity of the amino acids around the active site for PET	Ma et al. (2018)
PETase	*Ideonella sakaiensis*	PET	Plasmid: pET-21b; Host: *E. coli* BL21-CodonPlus (DE3) RIPL	-S131A: not detected -D177A: not detected -H208A: not detected -W130A: increased -W130H: increased -M132A: decreased -W156A: decreased -A180I: no change marked -Q90A: decreased -S185H: increased -S209F: decreased -W68L: decreased -Q153L: no change marked -R94A: decreased -N212A: decreased[b]	structure-guided site-directed mutagenesis in: the active sites; substrate binding pockets; the residues involved in stabilizing the rigidity of the active site	Liu et al. (2018a)
PETase	*Ideonella sakaiensis* 201-F6	PET; PEF	Plasmid: pET-21b(+); Host: *E.coli* C41(DE3)	-S238F/W159H: 4.13% higher[c]	site-directed mutagenesis to narrow the PETase active site: S238 to provide new π-stacking and hydrophobic interactions to adjacent terephthalate moieties: His159 to allow the PET polymer to sit deeper within the active-site channel	Austin et al. (2018)
PETase	*Ideonella sakaiensis* 201-F6	PET	Plasmid: pET32a; Host: *E. coli* XL1-Blue	-S131A: decreased -R103G: decreased -C174S: decreased -C210S: decreased -W156A: decreased -S185H: decreased -I179A: decreased -W130A: decreased -W130H: decreased -M132A: decreased -Y58A: 80.73% MHET production; TPA production decreased -T59A: full activity in producing MHET; TPA production decreased[d]	site-directed mutagenesis to determine apo- and complex crystal structures of PETase and to identify key residues requires for catalysis by, for instance, disruption intra-molecular disulfide bridges DS1 or substitution His residue in the corresponding position	Han et al. (2017)
PETase	*Ideonella sakaiensis*	PET	Plasmid: pET15b; pET15a; Host: *E. coli* Rosetta gami-B	-S160A: almost complete loss -D206A: almost complete loss -H237A: almost complete loss -Y87A: 5% hydrolytic activity -M161A: 52% hydrolytic activity -W185A: 5% hydrolytic activity	structural and site-directed mutagenesis in order to confirm the residues involved in enzymatic catalysis and substrate binding: three catalytic residues S160, D206 and H237 replacement with A; four subsite I residues Y87, W185, M161 and I208 replacement with A; three subsite II residues W159,	Joo et al. (2018)

(Continued on following page)

TABLE 1 | (*Continued*) Genetic modifications of enzyme structure for enhancement of biodegradable abilities towards plastic.

Wild-type enzyme/strain	Wild-type microorganisms	Substrate specificity	Vector and host	Improvement in activity	Mutatation's information	References
				-I208A: 46% hydrolytic activity -W159A: 8% hydrolytic activity -S238A: similiar hydrolytic activity -N241A: 18% hydrolytic activity -R280A: similiar hydrolytic activity -W159H: dramatically decreased -S238F: dramatically decreased -C203A/C239A: dramatically decreased[e]	S238, and N241 replacement with A, W159 and S238 residues replacement with H and F; deletion of additional disulfide bond	
cutinase Thc_Cut2	*Thermobifida cellulosilytica* DSM44535	PET	Plasmid: pET26b(+); Host: *E. coli* BL21-Gold(DE3)	-R19S: 3.4 fold -R29N: 17.6 fold -A30V: 17.0 fold -Q65E: decreased -L183A: 1.4 fold -R187K: 4.9 fold -double mutant R29N A30V: 8.4 fold -triple mutant R19S R29N A30V: 7.2 fold[a]	site-directed mutagenesis of amino acids located outside the active site on the Thc_Cut2 surface—exchange of selected side chains with the corresponding side chains of more active Thc_Cut1	Herrero Acero et al., 2013
cutinase	Fusarium solani pisi	PET	Plasmid: pET25b(+); Host: *E. coli* BL21 (DE3)	-L81A: 4.0 fold -L182A: 5.2 fold -N84A: 1.7 fold -V184A: 2.0 fold -L189A: decreased[f]	site-directed mutagenesis to create more space in the active site of the cutinase	Araújo et al. (2007)
cutinase Tfu_0883	*Thermobifida fusca*	PET	Plasmid: pET20b; Host: *E. coli* BL21 (DE3)	-I218A: 1.2 fold -Q132A/T101A: 1.6 fold[f]	site-directed mutagenesis to create space and to increase hydrophobicity of the catalytic side	Silva et al. (2011)
cutinase TfCut2	*Thermobifida fusca* KW3	PET	Plasmid: not mentioned; Host: *E. coli* BL21 (DE3)	-G62A: 4.0 fold	exchange of selected amino acid residues of active site in a substrate binding groove of TfCut2 with those present in cutinase LCC	Wei et al. (2016)
cutinase-type polyesterase (Cut190)	*Saccharomonospora viridis* AHK190	PET	Plasmid: pGEM-T; pQE80L; Host: *E. coli* DH5α; *E. coli* Rosetta-gami B (DE3)	-S226P: 1.4 fold -S226P/R228S: 2.1 fold -S226P/R228S/T262K: 2.2 fold[g]	cloning a putative cutinase gene (cut190); site-directed mutagenesis to substitute: S226 with P and R228 with the neutral S, T262 with K to enhance the salt-bridge formation	Kawai et al. (2014)
LC-cutinase	leaf-branch compost	PET	Plasmid: pHK; Host: *E. coli* DH5α; *E. coli* BL21 (DE3)	-*C. thermocellum* DSM1313:pHK-LCC: 62%[h]	insertion of the signal peptide sequence of cellulose Cel48S and a constitutive promoter of gene Clo1313_2638 (P_{2638}) to *Clostridium thermocellum* for the secretory production of LCC	Yan et al. (2020)

(Continued on following page)

TABLE 1 | (*Continued*) Genetic modifications of enzyme structure for enhancement of biodegradable abilities towards plastic.

Wild-type enzyme/strain	Wild-type microorganisms	Substrate specificity	Vector and host	Improvement in activity	Mutatation's information	References
LC-cutinase	leaf-branch compost	PET	Plasmids: pET21b(+); pET26b(+); Host: *E. coli* BL21 (DE3)	-WCCG: 90% in 10.5 h -ICCG: 90% in 9.3 h[i]	site-specific saturation mutagenesis in the first contact shell of groove; replacing the divalent-metal-binding site with a disulfide bridge; mutations to improve thermostability	Tournier et al. (2020)
LC-cutinase	leaf-branch compost	PET	Plasmid: PET28; PJ912; Host: *E. coli* BL21 DE3; *P. pastoris*	-LCC-NG -LCC-G: induction of aggregation 10°C higher than LCC-NG; improvement in the catalytic performance for PET hydrolysis; the rate of aggregation was found to be slower	site directed mutagenesis to introduce three putative N-glycosylation sites to improve LCC resistance for aggregation	Shirke et al. (2018)
cutinase TfCut2, LC-cutinase, carboxyl esterase TfCa	*Thermobifida fusca* KW3	PET	Plasmid: pET-20b(+); Host: *E. coli* BL21(DE3)	-TfCa/LCC: 47.9% weight loss/24 h -TfCut2/LCC: 20.4% weight loss/24 h[j]	site-directed mutagenesis for immobilization of TfCa on SulfoLink resin by addition an oligopeptide of G-S-C at the C-terminus of TfCa	Barth et al., 2016; Oeser et al., 2010
cutinase (Cut) and lipase (Lip)	*Thermomyces lanuginosus* (Lip); *Thielavia terrestris* NRRL 8126 (Cut)	PVAC; PCL	Plasmid: pPICZαA; Host: *E.coli* DH5α; *P. pastoris* KM71H	-Lip-Cut: 13.3, 11.8 and 5.7 times higher compared to Lip, Cut and Lip/Cut mixture, respectively	construction of chimeric lipase-cutinase (Lip-Cut) system overexpressed in *P. pastoris* to enhance the synergistic action of both enzymes	Liu et al., 2018b; Liu et al., 2019b
cutinase 1 (Thc_Cut1)	*Thermofida cellulosilytica*	PET, PBS, PHBV	Plasmid: pMK-T; pPICZαB; Host: *E. coli* XL-10 cells; *P. pastoris* KM71H	-Thc_Cut1_koAsn: no significant differences (PET)[d] -Thc_Cut1_koST: no significant differences (PET)[d]; 92% of weight loss (PBS)[K]	knock out of the three glycosylation sites at N29, N49, N161 (Thc_Cut1_koAsn) and S31, T51, S163 (Thc_Cut1_koST) by changing the nucleotide sequence to investigate the influence of glycosylation on the activity and stability	Gamerith et al. (2017)
polyhydroxybutyrate depolymerase (PA_PBM) and polyamidase (PA)	*Alcaligenes faecalis* (PA_PBM); *Nocardia farcinica* IMA 10152A (PA)	PUR	Plasmid: pET26b(+); Host: *E. coli* XL10-Gold; *E. coli* BL21	-fusion polyamidase PA_PBM: 4 fold	C-terminal fusion of a hydrophobic binding module of PA_PBM to PA to target the catalytic domain to the polyester interface more effectively	Gamerith et al. (2016)
Alkane hydroxylase	*Pseudomonas* sp. E4	LMWPE	Plasmid: pUC19; Host: *E. coli* BL21	-recombinant cell viable even after the biodegradation tests at 37 °C for 80 days	expression of alkane hydroxylase gene (*alkB*) in *E.coli* BL21 to mineralize LMWPE	Yoon et al. (2012)

[a]*expressed by kinetic parameters (kcat/KM).*
[b]*expressed by PET, degradation efficiency towards PET, bottle.*
[c]*expressed by the loss in the absolute crystallinity.*
[d]*expressed by the production levels of MHET, and TPA.*
[e]*expressed hydrolytic activity using BHET, as a substrate.*
[f]*expressed by released TPA, during hydrolytic activity towards PET.*
[g]*expressed as enzyme activity measured under standard conditions.*
[h]*expressed by the weight loss of PCL, films.*
[i]*expressed as enzymatic depolymerization of post-consumer PET, waste.*
[j]*expressed by PET, degradation efficiency towards PET, films.*
[k]*expressed by the weight loss of PBS, films.*

attack by the serine oxygen to the carbonyl carbon present in the ester bond. Negatively charged aspartate stabilizes positively charged histidine residue; thus the established charge transfer network enables serine to carry out a nucleophilic attack (Han et al., 2017). So far, homology has been found in the sequences of cutinases and PETase. Yoshida et al. (2016) observed 51%

similarity in amino acid sequence with the hydrolase present in *Thermobifida fusca* (TfH). Furthermore, similar to cutinase from *Fusarium solani*, PETase from *Ideonella sakaiensis* has two disulfide bridges that stabilize the structure of the enzyme molecule. Moreover, as was demonstrated before, an additional disulfide bond in PETase influences the thermal stability of the enzyme (Matak and Moghaddam, 2009; Joo et al., 2018). Phylogenetic analyses performed comparing these two enzymes have also revealed the presence of a highly conserved region recognized as a nucleophilic elbow that contains serine in the central part of the consensus sequence (Joo et al., 2018). Despite the similarities, an important difference is the width of the active site cleft, in comparison with cutinase from TfH; this slot is three times larger at its widest point in PETase (Austin et al., 2018). Furthermore, the residues surrounding the nucleophilic serine in the catalytic triad were found to be considerably different, which affects the substrate selectivity represented by these enzymes (Liu et al., 2018b).

Recently, many studies have been conducted to improve and better understand the mechanisms of action of these enzymes, especially PETase or MHETase (Oda 2021; Pinto et al., 2021). PETase is recognized as being responsible for hydrolytic conversion of PET into oligomers of mono-2-hydroxyethyl terephthalate (MHET), whereas MHETase hydrolyses MHET into terephthalic acid (TPA) and ethylene glycol (EG) (**Figure 2**). Thus, recombination and overexpression of those enzymes may be crucial for more efficient degradation of PET as well as monomer recycling (TPA and EG) and for bioconversion to high-value compounds (Furukawa et al., 2019; Taniguchi et al., 2019).

In mechanisms of enzymatic degradation of polyesters, such as PET, apart from plastic properties, the protein structure plays a key role. Especially, the regions on the surface outside the active site of the enzymes and binding modules are essential, both in interaction with the polymer and during the hydrolysis (Acero et al., 2013). For instance, Liu et al. (2018a) assumed in their study that the wide substrate-binding pocket of PETase is critical for PET hydrolysis (Liu et al., 2018b). In contrast, Austin et al. (2018) narrowed the binding cleft and observed improvement in PET degradation (Austin et al., 2018). It should also be emphasized that mutations are frequently used to create greater space in the active sites to fit the large, inaccessible polymer particles and to construct a more hydrophobic substrate-binding site (Araújo et al., 2007). Silva et al. (2011) noted that levels of adsorption to the PET surface are affected by the hydrophobic character of the enzyme active site (Silva et al., 2011). Thus, almost all modifications are related to the active sites of enzymes or their external part (**Table 1**). Unfortunately, during the creation of enhanced mutants, some obstacles arise. For instance, one of the difficulties emerging during enzymatic degradation of PET by PETase is the location of the enzyme inside the cells. It is perceived as a limiting factor in direct contact of PETase with the solid PET. The consequence is difficulty in establishing a high-throughput screening method in the evaluation of the hydrolysis rate by the modified strains. Fortunately, the solution is an application of developed cell-free protein-expression systems. The system is known as a useful tool in functional and structural proteomics for proteins that could not be expressed *in vivo* in bacterial cells. Furthermore, the system offers several advantages in comparison to traditional cell-based expression methods. First of all, it allows for easy modification of reaction conditions, shortening expression time or reducing the volume of reaction. The productivity exceeds hundreds of micrograms of protein per millilitre of reaction volumes. Interestingly, in the light of the PET problem mentioned above, application of a cell-free protein-expression system allows for the direct contact of expressed protein with solid PET, providing high-throughput screening of PET hydrolytic enzymes (Murthy et al., 2004; Katzen et al., 2005; Ma et al., 2018).

Engineering of PETase

In 2016 Yoshida et al. published a report about the newly isolated bacterium *Ideonella sakaiensis* 201-F6 that was able to use PET as its major carbon and energy source (Yoshida et al., 2016). Because knowledge of the protein structure is crucial for its further modifications, shortly afterwards many reports about *I. sakaiensis* PETase (IsPETase, EC 3.1.1.101) structure were published. It was shown that this enzyme is a hydrolase and possesses a strictly conserved active site with a Ser-His-Asp catalytic triad and contains an optimal substrate binding site to hold four mono(2-hydroxyethyl) terephthalate (MHET) moieties of PET. PETase enzyme exhibits an optimum pH range of 7–9 and the stability between pH 6 and 10 (Liu et al., 2019b). For purified PETase enzyme applied on PET film, pH 9.0 was identified as optimal, whereas the optimum temperature was estimated as 30°C (Han et al., 2017). PETase exhibits lower activity on *p*-nitrophenol-linked aliphatic esters in comparison to other cutinases, but towards PET the enzyme exhibits 5.5- to 120-fold higher activity compared to the other enzymes (Han et al., 2017; Joo et al., 2018). Attempts to improve the native PETase enzyme from *Ideonella sakaiensis*, which requires a mild environment for growth, are motivated by the relatively low stability of this enzyme. Introducing modifications to the amino acid chain may result in enhanced thermal stability by this protein and could help it maintain activity for a longer time (Joo et al., 2018). Mostly the enzyme's improvement is focused on site-directed mutagenesis. In the study of Joo et al. (2018) among 14 mutants, created by structural and site-directed mutagenesis, only the variant IsPETase^{R280A}, where the arginine (R) in position 280 was replaced with alanine (A), showed increased activity of PETase. The activity towards PET film as a substrate increased by 22.4% in 18 h and 32.4% in 36 h in TPA and MHET release in comparison to IsPETase$^{W/T}$. This mutant also expressed hydrolytic activity using BHET as a substrate at a similar level compared to the wild-type PETase (Joo et al., 2018).

The subsequent study focused on analysing the structure of the PETase enzyme molecule, comparing it to other ⍺/β-hydrolases enzymes, and performing the most promising modifications that could affect the thermal properties of this protein (Son et al., 2019). The possibility for enhancement of the PETase enzyme was the introduction of two mutations that, as previously, would allow the establishment of additional hydrogen bonds to stabilize the molecule. For this purpose, an IsPETase variant possesses

changes in serine (S) located at position 121 (to aspartic acid (D) or glutamic acid (E)) and aspartate (D) (to histidine (H)) in position 186 resulting in the S121D/D186H and S121E/D186H mutants have been established.

In other studies, Son et al. (2019) have applied a previous achievement of generating the PETase R280A mutant in the work of Joo et al. (2018) and introduced it to the S121E/D186H mutant described above. The study showed that the obtained triple mutant (S121E/D186H/R280A) degrades PET 13.9-fold better than the native protein and 2.3-fold than the previously established R280A protein variant.

Next, Dai et al. (2021) proceeded with further prospectively profitable changes to the structure of this protein variant. The changes in the enzyme were based on the addition of hydrophobic substrate-binding domains such as CBM (cellulose-binding domain), PBM (poly(3-hydroxybutyrate)) binding domain and HFB4 (hydrophobin) to the C-terminus end of PETase. Authors supposed that the presence of CBM, PBM or HFB4 domain in the protein structure could improve the enzyme binding to the hydrophobic surface of PET molecules, which would be associated with an enhanced level of plastic degradation by these mutants. The effect of the implemented modifications was tested based on the amount of PET degradation products (TPA and MHET) released during the incubation with the novel mutants compared to IsPETase D121E/D186H/R280A (IsPETaseEHA). The experiments showed that among the three obtained mutants (IsPETaseEHA_CBM, IsPETaseEHA_PBM, IsPETaseEHA_HFB4), only the protein containing an additional CBM domain improves PET degradation. Increase in PET breakdown products concentration was 2.28-fold increased in comparison to the original mutant and was 251.5 μM of total hydrolysis products. The two remaining variants significantly reduced PET degradation capacity (Dai et al., 2021).

In the study Han et al. (2017) created 12 mutants in order to identify key residues required for catalysis, most of them showed decreased activity in production levels of MHET and TPA compared to the wild-type PETase. Only variant Y58A (possessing change in tyrosine (Y) at position 58 to alanine (A)) exhibited 80.73% MHET production compared to MHET released by wild-type PETase and T59A, which showed full activity in producing MHET. However, in both cases, TPA production decreased (Han et al., 2017). Ma et al. (2018) aimed to create novel high-efficiency PETase mutants through mutagenesis of six key residues around the substrate-binding groove of PETase. By application of a rapid cell-free screening system, they obtained three mutants. In comparison with wild-type PETase, the R61A (exchange in arginine (R) to alanine (A)), L88F (leucine (L) in position 88 changed to phenylalanine (F)), and I179F (isoleucine (I) exchanged to phenylalanine (F)) mutants exhibited 1.4, 2.1 and 2.5 fold increases in the enzymaticaffinity to PET, respectively. The strongest catalytic activity expressed by TPA concentration and by weight loss of PET film incubated with purified enzyme was shown by the I179F mutant (6.38 mmol L^{-1} of released TPA after 48 h of incubation and 22.5 mg per μmol·L^{-1} PETase per day). L88F and R61A mutants reached 17.5 and 13.5 per μmol·L^{-1} PETase per day, respectively, whereas the degradation rate of wild-type PETase was only 8.2 mg per μmol·L^{-1} PETase per day. Furthermore, scanning electron microscopy (SEM) was used to observe the changes in the morphology of the PET film surface after treatment with the I179F mutant in comparison to the negative control. The surface of the PET film was roughened and eroded, and a large number of holes were observed (Ma et al., 2018). In the study of Liu et al. (2018b) structure-guided site-directed mutagenesis was used to improve PETase catalytic efficiency. Several mutants were created with mutations in the active sites, substrate binding pockets or in the residues involved in stabilizing the rigidity of the active site. The hydrolytic activity of PETase was analysed with respect to BHET. Only two mutants described as W130H, where tryptophan (W) has been replaced by histidine (H) and S209F possessing serine (S) exchange to phenylalanine (F), showed increased activity. Interestingly, the authors performed a PETase activity assay on PET drinking bottles. Similarly, only two mutants, described as W130A and W130H, had higher hydrolytic activity towards PET bottles in comparison to unmodified PETase (Liu et al., 2018a). However, PETase retains the ancestral α/β-hydrolase fold with a core consisting of eight *ß*-strands and six *α*-helices and exhibits a more open active-site cleft than cutinases. Austin et al. (2018) narrowed the binding cleft *via* site-directed mutagenesis of two active-site residues and surprisingly observed improved PET degradation. They created the double mutant S238F/W159H (with serine (S) in position 238 replaced with phenylalanine (F) and tryptophan (W) in position 159 exchanged to histidine (H)) that altered important substrate-binding interactions. The S238 mutation provided new *p*-stacking and hydrophobic interactions to adjacent terephthalate moieties, while the conversion to His159 from the bulkier Trp allowed the PET polymer to sit deeper within the active-site channel. Moreover, in the study, it was demonstrated that the mutant could degrade polyethylene-2,5-furandicarboxylate (PEF), which is a PET replacement. The results suggested that PETase is not fully optimized for crystalline PET degradation (Austin et al., 2018).

Another modification of PETase was performed using the Premuse tool(Meng et al., 2021), by which the selected putative mutations in the protein structure could correspond to natural future evolution in the amino acid chain of the protein. A thorough in-silico analysis highlighted the potential positive effect of the W159H/F229Y mutation to boost the catalytic capacity of PETase. The newly obtained PETase double mutant having modified tryptophan (W) at position 159 to histidine (H) and phenylalanine (F) at position 229 to tyrosine (Y) showed higher thermal stability compared to the wild-type enzyme and the single variants of the mutant proteins (W159H and F229Y). The authors indicated that IsPETase W159H/F229Y after 24 h reaction at 40 °C resulted in a 40-fold increased amount of degradation products in comparison with the native enzyme, however, the authors did not report the specific values of the obtained concentrations of the released compounds (Meng et al., 2021).

Engineering of MHETase

The MHETase discovery occurred at a similar time as PETases, but it is not as well studied an enzyme as PETase despite the fact that they are cooperatively responsible for the degradation of PET by Ideonella sakaiensis 201-F6 (Tanasupawat et al., 2016). Structurally, MHETase is an α/β hydrolase that exhibits high substrate specificity and its catalytic triad is formed by S225-H528-D492. Additionally, the domain arrangement is similar to those observed in feruloyl esterases but in contrast to them, MHETase exists as a monomer instead of a dimeric structure (Sagong et al., 2020). MHETase possesses optimum temperature at 45°C and a wide range of pH activity between 6.5–9.0 (Palm et al., 2019). Similar to other hydrolases, MHETase performs a nucleophilic attack on the carbonyl carbon via serine (Pinto et al., 2021).

The metabolic engineering of MHETase is not yet as strongly advanced as that of PETase described in detail in the previous section. Nevertheless, we can highlight several examples of previous studies in which modifications in the amino acid sequence of this protein have been undertaken. One of the earliest MHETase mutagenesis was carried out during the work on the determination of its exact structure and involved an amino acid change within the active site of the enzyme. Palm et al. (2019) have generated a number of mutants to identify key amino acid residues in terms of enzyme activity. Their study revealed that one of the key amino acids responsible for substrate binding is Phe495, whose replacement with alanine (A) resulted in the formation of the F495A protein variant. Studies of catalytic properties of this mutant have shown that the turnover rate of MHET compared to the wild-type enzyme was more than 2 times lower and was about 5 s-1 (Palm et al., 2019).

The subsequent study involving engineering MHETase to enable its degradation of BHET was conducted by Sagong et al. (2020). Investigations performed by these researchers indicated that MHETase can bind to BHET as substrate, however, the hydrolysis activity is very low. Studies with targeted mutagenesis indicated an important role of hydrophobic residues Leu254, Trp397, Phe415 and Phe495 in substrate binding and enzymatic catalysis. Sagong et al. (2020) have performed several mutations that significantly affected BHET binding by the created mutants. All of them were based on mutagenesis at phenylalanine position 424 (F424), and for three mutants (F424N, F424V, and F424I), a significant, more than 3-fold increase in activity against BHET relative to native MHETase was observed. Additional arginine point mutation at position 411 to lysine (R411K) was also found to result in a 1.7-fold increase in activity against BHET substrate compared to the wild-type enzyme. Based on the results, further mutagenesis was performed incorporating the revealed properties of the single mutants, which resulted in the formation of double protein variants (R411K/F424N, R422K/F424V, and R411K/F424I). The relative activity to BHET for the resulting mutants was 8.7, 10.5 and 11.1%, respectively than the native MHETase possessing 1% relative activity towards this substrate. Further studies on the MHETase mutants were conducted based on a previous report by Palm et al. (2019) in which an important role for the S416A mutation was identified. The resulting R411K/S416A/F424I triple mutant was shown to be 15.3-fold more active against BHET than wild-type MHETase. Activity assays against amorphous PET film were performed on the triple mutant in two variants: without prior hydrolysis of IsPETaseEHA and after incubation with the modified PETase enzyme (see the paragraph on PETase protein engineering above for a detailed description of this mutant). As expected, neither the wild-type MHETase nor the enhanced triple mutant showed activity against PET films without prior IsPETaseEHA pre-treatment. Interestingly, the researchers found that with the use of PET film pre-treated for 10 days with IsPETaseEHA, both the wild-type MHETase enzyme and the triple mutant R411K/S416A/F424I showed activity against PET film. Specified values obtained in this study after 72 h was about 8 μM of released degradation products by mutant variant, while for the control (wild-type MHETase) it was 4 μM (Sagong et al., 2020).

Modification of Cutinases

Cutinases (EC 3.1.1.74) are similar to PETase. They belong to the α/β hydrolases group and possess the classical catalytic triad Ser-His-Asp. In nature, cutinases are produced by plant pathogens to hydrolyse the polyesters of the cutin and the suberin layers. In addition, cutinases are able to catalyse reactions with various polyesters and other substrates such as long-chain triacylglycerols or waxes (Nyyssola, 2015). Cutinases possess a wide spectrum of pH optima, where most prefer neutral or alkaline pH. For the thermophilic bacteria Thermobifida fusca, researchers indicate a range of pH at 6.8–9 with optimum pH at 8.0 at an optimum temperature of 50–55 °C (Acero et al., 2011; Hegde and Veeranki, 2013). In the case of fungal cutinase using the example of cutinase from Fusarium solani, the optimum enzyme condition was determined in the range of pH 7.5–10 (Chen et al., 2008; Baker et al., 2012) and the optimum temperature range for this cutinase has been indicated at 25°C (Baker et al., 2012), 30°C (Chen et al., 2008) and 40°C (Pio and Macedo, 2009).

Since cutinases are universal and efficient esterases, their modification toward PET degradation has been done (Acero et al., 2013). The effect of site-directed mutagenesis, which exchanges selected surface-located amino acids between two polyester hydrolases from *thermobifida cellulosilytica* DSM44535, has been studied. As a result, six single mutants, one double mutant and one triple mutant were obtained. The degradation level of amorphous PET films was tested by enzymatic hydrolysis with the use of derived cutinases and quantification of the released degradation products (TA-terephthalic acid and MHET-mono-(2-hydroxyethyl) terephthalate). Incubation of PET with unmodified cutinase Thc_Cut2 as a control was provided. PET hydrolysis was performed for 2 days at 50°C at pH 7.0 with the 200 μgmL-1 of enzyme on pre-washed PET films with Triton-X 100 (Acero et al., 2013). The pre-treatment of non-ionic surfactant used in this study may lead to a decrease in the hydrophobicity of the polymer surface and consequently facilitate the binding of the enzyme with the substrate (Caparanga et al., 2009; Mohanan et al., 2020). Kinetic parameters for the mutants compared to the Thc_Cut2 (9 s-1mM-1) were performed, as a result, mutants carrying Arg29Asn (15 s-1mM-1) and/or Ala30Val (153 s-1mM-

1) exchanges showed considerably higher specific activity and higher kcat/KM values on soluble substrates. However, it should be noted that a triple mutant enzyme with Arg19Ser introduction negatively influenced all the parameters (Acero et al., 2013). Experiments performed on PET film, based on the measurement of TA and MHET released during hydrolysis showed that there is no significant increase in MHET concentration. However, an increased amount of TA released during PET degradation compared to Thc_Cut2 occurred for Ala30Val, Arg29Asn_Ala30Val and Arg19Ser_Arg29Asn_Ala30Val mutations. The highest TA concentrations measured in this experiment were 400 and 370 mM for Arg29Asn_Ala30Val and Arg19Ser_Arg29Asn_Ala30Val, respectively. Interestingly, the introduced Gln65Glu mutation resulted in a 36% decrease in the concentration of the amount of breakdown products for 3PET and completely inhibited PET degradation, despite the fact that kinetic parameters did not remarkably differ compared to the Thc_Cut2.

In other studies, a cutinase from *Fusarium solani pisi* was genetically modified to enhance its enzymatic activity. Site-directed mutagenesis targeted the region near the active site and as a result, two mutants with enhanced activity towards polyester fibres were obtained, named L81A and L182A. They showed an activity increase of four- and five-fold, respectively, when compared with the wild type, for PET fibres. The authors explained the increase in activity of these mutations by higher stabilization of TI and better accommodation of the substrate (Araújo et al., 2007).

Another successful improvement of the enzymatic degradation of PET was presented in the study of Silva et al. (2011). The active site of cutinase Tfu_0883 from *Thermobifida fusca* was modified by site-directed mutagenesis to increase the affinity of cutinase to PET and the ability to hydrolase it. The mutation I218A (isoleucine (I) replacement to alanine(A)) was designed to create space and the double mutation Q132A/T101A possessing glutamine (Q) and tyrosine (T) replaced with alanine (A) was designed both to create space and to increase hydrophobicity. The activity of both single and double mutants exhibited considerably higher hydrolysis efficiency towards PET fibres—a double mutant exhibited 1.6-fold increased hydrolysis activity (Silva et al., 2011).

In a similar study conducted by Wei et al. (2016), mutagenesis was used to increase the activity of the cutinase TfCut2 from *Thermobifida fusca*. By exchanging selected amino acid residues of the active site in a substrate-binding groove of TfCut2 with those present in LCC, mutants with increased PET hydrolytic activity were obtained. The most active mutants were G62A, possessing glutamine (G) replaced by alanine (A) and G62A/I213S where additional exchange of isoleucine (I) by serine (S) was done. As a result, a 2.7-fold increase in weight loss of PET films was obtained compared to the wild-type enzyme. Moreover, kinetic analysis based on the released PET hydrolysis products confirmed the superior hydrolytic activity of G62A with a fourfold higher hydrolysis rate constant and a 1.5-fold lower substrate-binding constant than those of the wild-type enzyme (Wei et al., 2016). Next, the mutant TfCut2 G62A obtained by Wei et al. (2016) was a subject of the interesting study of Furukawa et al. (2019). In the study, it was found that low-crystallinity PET (lcPET) hydrolysis may be increased by the addition of a cationic surfactant that attracts enzymes near the lcPET film surface via electrostatic interactions. This approach was applicable to the mutant TfCut2 G62A/F209A and wild-type TfCut2. As a result, the degradation rate of TfCut2 G62A/F209A in the presence of the cationic surfactant (dodecyl trimethyl ammonium) increased 12.7 times over that of wild-type TfCut2 in the absence of the surfactant. A positive effect of surfactant addition was evident for the native enzyme as well as all mutants used except H129E/F209S. The long-duration reaction showed that lcPET film had the fastest biodegradation rate of lcPET film so far (97 ± 1.8% within 30 h) (Furukawa et al., 2019). It was also noted that the addition of a cationic surfactant, as well as the increased reaction temperature, results in enhanced hydrophobic interactions between the enzyme and the plastic surface, and consequently increases the amount of enzyme bound to the lcPET surface (Furukawa et al., 2019). Moreover, higher temperature raises the mobility of the polymer chain, which further facilitates the binding of the enzyme to the substrate (Ronkvist et al., 2009).

Tournier et al. (2020) found that leaf-branch compost cutinase (LCC) demonstrated the highest thermostability and was at least 33 times more efficient than other enzymes tested in their study. In differential scanning fluorimetry experiments it was shown that LCC is thermally stabilized in the presence of calcium ions. To avoid salt supplementation, the authors focused on improving the activity and thermostability of LCC by enzyme engineering. By using the alternative strategy of replacing the divalent metal binding with a disulfide bond the researchers obtained thermal stabilization of LCC without dependence on calcium ions. Moreover, by site-direct saturation mutagenesis, they tested 209 mutants. Most of the modified variants showed less than 1% specific activity in comparison to the wild-type LCC, but the F243I and F243W mutations, in which phenylalanine (F) at position 243 was replaced with isoleucine (I) or tryptophan (W), showed elevated activity. The obtained cutinase variants gained specific activity by 27 and 18%, respectively. Finally, they obtained an enhanced PET hydrolase that was able to depolymerize over 90% PET into monomers in over 10 h (10.5 and 9.3 h for mutants WCCG (F243W/D238C/S283C/Y127G) and ICCG (F243I/D238C/S283C/Y127G), respectively with the use of 3 mg of enzyme per 1 g of PET. The productivity of ICCG mutant was determined at 16.7 g of terephthalic acid per litre per hour at 72°C, which is a 98-fold increase compared to TfCut2 investigated before (Wei et al., 2019). Wild type LCC enzyme achieved only 53% of conversion after 20 h, which corresponds with its lower thermostability in comparison to the mutants. Although X-ray crystallography showed no substantial difference between parental LCC and ICCG, molecular-dynamic simulations revealed that mutations introduced in ICCG facilitated the catalytic binding of 2-HE(MHET)3 compared with parental LCC (Tournier et al., 2020).

Kawai et al. (2014) created a Cut190 (S226P/R228S), a double mutant enzyme for PET degradation. They cloned the cutinase gene (cut190) from *Saccharomonospora viridis* AHK190 and

expressed it in *Escherichia coli* Rosetta-gami B (DE3). It was observed that the substitution of Ser226 with Pro and Arg228 with Ser yielded the highest activity and thermostability of the new enzyme. Also, they noted that the presence of the Ca^{2+} ion enhanced the enzyme activity and thermostability in comparison to both the wild-type enzyme and mutant Cut190. Circular dichroism suggested that the Ca^{2+} changes the tertiary structure of Cut190 (S226P/R228S) (Kawai et al., 2014). High-level expression of LCC was also achieved due to insertion of the signal peptide sequence of cellulose Cel48S and a constitutive promoter of the gene Clo1313_2638 (P2638) in *Clostridium thermocellum*. Improved degradation of commercial PET films was observed and maximum weight loss (approximately 62%) was achieved after 14 days of incubation at 60°C (Yan et al., 2020).

Genetic engineering may also be a solution to many problems related to the stability of enzymes. For instance, Shirke et al. (2018) underlined that aggregation is emerging as a major factor that reduces LCC kinetic stability. In its native state, LCC is highly prone to aggregation owing to electrostatic interactions. Since LCC precipitates even at room temperature and low concentrations, the purification and storage of enzymes require salt concentrations that vary with protein concentration. Moreover, efficient PET hydrolysis requires a temperature around 70°C, which is very close to the temperature of LCC structure loss. To overcome these problems, Shirke et al. (2018) proposed the expression of native LCC in *Pichia pastoris*, resulting in the production of glycosylated LCC (LCCG). They introduced three putative N-glycosylation sites, which improved resistance to aggregation even at high-temperature conditions, leading to a 10°C increase in the thermal aggregation point and a significant increase in kinetic stability. Furthermore, glycosylation resulted in improved catalytic PET hydrolysis (Shirke et al., 2018). On the other hand, Gamerith et al. (2017) aimed to investigate the influence of glycosylation on the activity and stability of cutinase 1 (Thc_Cut1) from *Thermobifidia cellulosilytica*. They expressed Thc_Cut1 and two glycosylation site knockout mutants, Thc_Cut1_koAsn and Thc_Cut1_koST, in *P. pastoris*. However, the created mutants hydrolysed aromatic (PET) and aliphatic (PHBV and PBS) polyester powders at very different rates based on quantification of released products by HPLC. Thc_Cut1_koST was the most effective among all enzymes. The highest TPA yield was obtained for Thc_Cut1_koST mutant, which caused hydrolysis of 24% of the starting PET powder amount. Due to the fact that the Thc_Cut1_koST mutant exhibited higher protein production yield in engineered *P. pastoris* yeast, this variant was used in further studies on PHBV and PBS degradation. The authors did not observe significant differences in PHBV degradation between Thc_Cut1 and Thc_Cut1_koST mutants, due to the similar amounts of 3-HBA (3-hydroxybutyric acid) at around 0.5 mM after 96 h of incubation. A similar trend was observed by investigating a PBS film weight loss, where a 92% decrease in the mass of polymer film was obtained for the applied mutant and 41% for Thc_Cut1 within 96 h of hydrolysis (Gamerith et al., 2017).

Although the main focus in genetic engineering of microorganisms and enzymes with biodegradation activity is directed towards PET, some studies present results for other plastics. For instance, the alkane hydroxylase gene (alkB) from *Pseudomonas* sp. E4 was expressed in *E. coli* BL21. A recombinant strain secreted recombinant alkane hydroxylase (AH) and was able to mineralize 19.3% of the low molecular weight polyethylene (LMWPE) to CO_2 after incubation in the compost for 80 days at 37°C, while the recipient cell was not active at all toward LMWPE biodegradation (Yoon et al., 2012).

Synergistic Activity of Chimeric Enzymes

Chimeric enzymes, also known as fusion proteins, are proteins formed by combining two or more unrelated genes that originally encoded distinct proteins. The resulting proteins exhibit the attributes of all the proteins used in the fusion and constitute a single, combined molecule. Suitably designed hybrid proteins offer many opportunities due to their wide range of properties and can be used in many fields (Yu et al., 2015). Thus, the application of multiple enzyme systems for the biodegradation of plastic seems to be a very promising solution. It is reasonable to assume that enzymes might be used synergistically with other enzymes in polymer degradation due to the complementary properties of both enzymes in both catalysis pattern and substrate specificity (Liu et al., 2019b). A prime example is the connection of two enzymes in the biodegradation of PET. It is known that during PET degradation, accumulating MHET is an important factor that limits the efficiency of hydrolysis. To avoid this problem, the recombinant expression and purification of TfCut2 from *Thermobifida fusca* KW3 and LC-cutinase (LCC) were proposed. In the study of Barth et al. (2016) the dual system was LCC or TfCut2 combined with immobilized TfCa on the SulfoLink resin—which was generated by the addition of oligopeptide of glycine-serine-cysteine at the C-terminus via site-directed mutagenesis. The introduction of the C-terminal oligopeptide did not cause a significant reduction in its hydrolytic activity against *p*-NPB, BHET and MHET. The immobilized enzyme maintained approximately 94% of its initial activity at 60°C, whereas free TfCa resulted in a complete loss of activity at 55 °C. Moreover, the usage of a dual enzyme reaction system with LCC or TfCut2 caused a 47.9% or 20.4% weight loss, respectively, of the PET films after a reaction time of 24 h (Barth et al., 2016). An artificial chimeric enzyme was also constructed by Liu et al. (2018a). In their study lipase (Lip) from *Thermomyces lanuginosus* and cutinase (Cut) from *Thielavia terrestris* NRRL 8126 were used for the construction of bifunctional lipase-cutinase (Lip-Cut) by end-to-end fusion and overexpression in *Pichia pastoris* (Liu et al., 2018b). Lip-Cut exhibited a more efficient degradation ability towards poly(ε-caprolactone) (PCL). The weight loss of PCL films was 13.3, 11.8, and 5.7 times higher (at 6 h) than those obtained by Lip, Cut and the Lip/Cut mixture, respectively. GC-MS analysis revealed that the main products produced during hydrolysis were 6-hydroxyhexanoic acid and 3-caprolactone. Moreover, SEM analysis showed that the surface of the PCL film became rougher and more holes were observed after 4 h of treatment with bifunctional Lip-Cut than in the case of other enzymes after 48 h (Liu et al., 2019a). Gamerith et al. (2016), using C-terminal fusion, fused a hydrophobic binding module of polyhydroxybutyrate depolymerase (PA_PBM) from

Alcaligenes faecalis to polyamidase (PA) from *Nocardia farcinica* IMA 10152A. The fusion polyamidase (PA_PBM) indeed resulted in a more active enzyme on commercial polyurethane copolymers as indicated by the release of 4,4′-diaminodiphenylmethane (MDA) and different oligomers (Gamerith et al., 2016).

An interesting improvement of PET degradation was the two-enzyme system described by Knott et al., 2020. In that study, the authors constructed an MHETase: PETase chimeric protein covalently linking the C-terminus of MHETase to the N-terminus of PETase of varying glycine-serine linker lengths (8, 12 or 20 aa residues). All chimeric proteins exhibit improved PET and MHET turnover relative to the free enzymes. Hydrolysis of amorphous PET incubated with 0.25 mg of PETase and 0.5 mg of MHETase per gram of PET resulted in the release of 0.25 mM MHET and TPA by PETase and 0.45 mM MHET when co-incubated with the two enzymes. The use of the MP8, MP12 and MP20 chimeras increased the amount of TPA released threefold (1.4, 1.45, and 1.5 mM, respectively). Interestingly, the chimeric constructs linking the C-terminus of PETase to the N-terminus of MHETase were not capable of expressing the protein (Knott et al., 2020).

Another enhancement of PETase based on the implementation of hybrid proteins to increase its activity and thermal stability was performed by Chen et al. (2021). The work conducted by these researchers involved the addition of an amino acid chain containing glutamic acid (E) and lysine (K) to the C-terminus of the PETase enzyme, which resulted in the formation of different PETase-EK fusion protein variants (5, 10 and 30 kDa). Thermal stability assays performed for the obtained mutants compared to the native PETase showed that each of the obtained proteins exhibited better stability (80% activity after 6 h incubation at 40°C) than the native enzyme (65% activity after incubation under the same conditions). The ability to degrade PET was verified by the number of released degradation products during incubation with amorphous PET film and PET bottle film. The experiment demonstrated that each mutant caused a significantly higher level of PET degradation (on both plastic type materials used) compared to the wild-type enzyme. The best results were obtained with PETase-EK30, which after 6 days of incubation at 40°C resulted in the release of 302.4 and 146.2 μM total MHET and TPA after incubation with amorphous PET film and PET bottles, respectively. The incubation with the native enzyme resulted in the release of 32.8 and 13.9 μM of MHET and TPA for the appropriate substrates, correspondingly. It is worth noting that for the native enzyme, from day 1 of incubation to day 6, the amount of products released did not change significantly. In the case of the mutants, a progressive increase in the amount of breakdown products was noted from day 1 to day 4 of incubation, while between days 4 and 6 the measured concentrations were at equivalent amounts (Chen et al., 2021).

Prospective Applications of Modified Microorganisms and Engineered Proteins in PET Waste Management

Since 1964 production of plastic has increased twentyfold, but almost 50 years after the introduction of the recycling process, only 14% of plastic packing is collected for reuse. PET used in bottles has the highest recycling rate, but globally only 7% of it is recycled bottle-to-bottle. Most of the plastic products after a single use are landfilled, and 32% escape the collection system to the natural environment (Ellen MacArthur Foundation and World Economic Forum, 2014; http://www3.weforum.org/docs/WEF_The_New_Plastics _Economy.pdf). The growing amount of plastic waste has forced the scientific community to look at this global issue. So far the published reports have shown that naturally isolated microorganisms possess a limited capability for plastic degradation, and it might take decades before microbes adapt to use plastic as a carbon source. The published data on genetically modified microorganisms or chemically engineered enzymes suggest that this direction offers a promising method for plastic waste management. Nowadays, through the chemical engineering of enzymes such as modification of the active site or by introducing new bonds, we can avoid cofactor supplementation with the simultaneous multifold improvement of their activities, reduction of the reaction time and increase of their thermal stability. The latter factor might be crucial since hydrolysis of PET needs a higher temperature than the glass transition temperature (Tg), which is 67–81 °C. A highly interesting approach that can be applied to PET degradation technology in the future is the concept of nano-immobilization of enzymes to improve their tolerance to temperature and pH. The first attempts to use immobilized enzymes have been recently achieved successfully (Jia et al., 2021). Another perspective, which should be included in the discussion, is the possibility of reuse of the products of PET degradation (such as EG and TPA) to synthesize a new PET with similar properties as a virgin PET. This will result in reduced demand for fossil substrates for plastic synthesis and simultaneously may be a branch where the use of immobilized enzymes can bring additional benefits. Prospectively, due to restrictions and concerns over the use of GMOs worldwide, safer and easier to safely handle modified proteins may become an alternative to the long-known bioremediation or biological recycling, which seems to be a distant goal when using GMMs. The application of enzymatic recycling with the use of enhanced mutants of PETase cutinase may be one solution in the future to reduce the level of contamination, and to our knowledge, such application is one of the latest developments in this field (Singh et al., 2021).

An interesting attempt was made by Roberts et al. (2020), where a microbial consortium of *Pseudomonas* and *Bacillus* species was applied to synergic PET degradation. Such a study may bring additional perspective about applying an enzymatic cocktail as a possibility to provide ester bond hydrolysis in PET formulated with engineered hydrolases. Despite the fact that microorganisms able to produce PET-degrading enzymes and capable of growing degradation products as the sole carbon source can be found in nature, the efficiency of PET hydrolysis is generally low. The other perspective that can be taken into consideration in further application attempts of genetic engineering and protein modification methods is related to the successful attempt to obtain microalgae capable of producing PETase enzyme (Kim et al., 2020). The use of an improved mutant of this protein in similar studies could

greatly enhance the research conducted in this area and bring interesting adaptive solutions for photosynthesizing eukaryotes.

In the future probably we will be able to use plastic wastes as a low-cost substrate for genetically modified microorganisms to produce value-added products such as enzymes, fatty acids, organic acids and others. As emphasized in this review, genetically modified microorganisms are a promising alternative for the plastic circular economy.

CONCLUSION

As the production of plastics is still increasing, enzymatic hydrolysis of PET and other plastic is gaining importance and interest from researchers. This way of degradation of plastic is evaluated as an environmentally friendly, novel strategy for the recycling of post-consumer plastic materials. Thus, in order to better adapt the enzymes to synthetic polymers, the use of genetic engineering may be a key to solving the plastic pollution problem. However, the engineering of novel hydrolases exhibiting highly efficient and specific catalytic properties towards PET materials remains a challenge. All findings presented above may provide further options to obtain effective enzymes for biocatalytic plastic recycling processes.

AUTHOR CONTRIBUTIONS

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Efficient biodegradation of aliphatic polyester by genetically engineered strains of the yeast *Yarrowia lipolytica*

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ABSTRACT

The increased production and consumption of plastic materials in today's world has caused a huge number of environmental problems, the solution of which is now crucial to improving the state of nature of our planet. In this study, we expressed cutinases from Fusarium solani f. sp. pisi and from Trichoderma reesei, individually or in combination with overexpression of the native lipase Lip2 in the yeast *Yarrowia lipolytica*. The engineered strains showed biodegradation activity toward polyester in the pH range between 4.0 and 9.0. The highest esterase activity was observed at pH 9.0 for the strain overexpressing cutinase from F. solani and lipase from Y. lipolytica, it reached 63.5 U ml^{-1}, moreover this strain was capable to degrade 0.5 g of polycaprolactone film within 144 h of shake flask culture. The obtained results showed that natural capability of Y. lipolytica combined with metabolic engineering results in a highly efficient biodegradation process.

1. Introduction

Plastic production has increased every year since 1950, and it reached 359 million tonnes in 2018 (PlasticsEurope Market Research Group (PEMRG)/Conversio Market & Strategy GmbH, 2019). Despite the fact that plastics have revolutionized the global industry in every field this increase has had a negative impact on the global ecosystem. Recently, large-scale research has focused on finding a solution to the ever-increasing level of plastic pollution (Ilyas et al., 2018). The research focuses on the biodegradation of plastic by microorganisms and improvement of this process. The capability of plastic biodegradation by microorganisms depends on the type of material, its surface, size and physicochemical properties (Sarjit et al., 2015). In the natural environment, factors such as the hydrophobicity and surface irregularities of plastic particles affect the adhesion of microorganisms on the surface of material (Howell and Behrends, 2006).

Plastics have been characterized as biodegradable, i.e., those that can be completely degraded by microorganisms and non-biodegradable. Poly-ε-caprolactone (PCL) is a biodegradable aliphatic polymer with the melting point at 59–60 °C. The biodegradation time of this polymer is 2–5 years in the external environment (Cameron and Kamvari-Moghaddam, 2012). Many studies have been carried out to investigate the degradability of PCL material by microorganisms. The decomposition of PCL has been proven by cutinase-producing microorganisms such as F. solani (Shi et al., 2020) and Cryptococcus (Ghosh et al., 2013). It was shown that cutinases from Humicola insolens, Pseudomonas mendocina, F. solani, Thermobifida cellulosilytica and Thermobifida fusca are able to hydrolyze aromatic polyesters such as PET (Herrero Acero et al., 2011). Polyethylene terephthalate (PET) is non-biodegradable, one of the most common aromatic polyester with annual worldwide production reaching 50 million tonnes (Bornscheuer, 2016).

Microbial degradation of plastic materials by different strains of bacteria, fungi and yeast is possible due to enzymes from the hydrolase class such as cutinases, lipases and PETases. In addition, interesting results were obtained using nitrifying bacteria, which produce significantly less extracellular polymeric substances (EPS) and soluble metabolic products (SMP) compared to the production of these compounds by heterotrophs (Sepehri and Sarrafzadeh, 2018). Many genetic modifications have been made to improve natural abilities of microorganisms for plastic degradation (Liu et al., 2018) or by overexpression of the enzymes involved in this process in the heterologous host (Gamerith et al., 2017; Ribitsch et al., 2011). A suitable host for heterologous protein expression is the yeast *Yarrowia lipolytica*. This is a well-known unconventional non-pathogenic yeast. Due to the ability to assimilate

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various hydrophobic carbon sources, such as n-alkanes, fatty acids or polyols, *Y. lipolytica* is used in the bioremediation processes of soil contaminated with oils, fuels or aromatic compounds. To date there has been done many research about environmental applications of Y. lipolytica. Vasiliadou et al., in 2017 noted the ability of *Yarrowia lipolytica* yeast to produce lipids with altered fatty acid content. Additionally, authors indicated the ability of this yeast to assimilate polyunsaturated fatty acids, which can be used in the treatment of hydrophobic wastes and food processing wastewater with high content of oils (Vasiliadou et al., 2018). Other studies have shown the ability of *Yarrowia lipolytica* to grow on medium supplemented with olive mill wastewater (OMW), characterized by a high chemical and biological oxygen demand and a high concentration of phenolic compounds (Sarris et al., 2017). Another study conducted on a medium with OMW, indicated that Y. lipolytica can grow on this waste after dilution to appropriate concentrations of phenolic compounds. Furthermore, *Yarrowia lipolytica* with appropriate supplementation can utilize this waste for the production of biomass, mannitol and citric acid and effectively remove phenolic compounds (Dourou et al., 2016).

Y. lipolytica produces native lipases, enzymes from the subclass of esterases which enable them to utilize triacylglycerides and might be helpful in hydrolysis of ester bonds in plastic polymers (Miller and Alper, 2019). Moreover, it possesses a wide range of molecular tools, which allows for efficient genetic modification (Abdel-Mawgoud et al., 2018; Holkenbrink et al., 2018; Schwartz et al., 2016; Wong et al., 2017). Due to these properties, Y. lipolytica is a promising microorganism in biodegradation of plastic materials.

In this study, we aimed to degrade plastic using genetically engineered strains of Y. lipolytica. Here, we functionally overexpressed heterologous genes encoding cutinases from Fusarium solani f. sp. pisi and Trichoderma reesei and the native Y. lipolytica lipase Lip2. The catalytic triad of both cutinases is composed of Ser-His-Asp amino acids (Roussel et al., 2014). The catalytic triad of cutinase from Fusarium solani f. sp. pisi includes Ser 120, His 188 and Asp 175 (Baker et al., 2012). The cutinase from the T. reesei catalytic triad consists of Ser 164, His 229 and Asp 216 (Roussel et al., 2014). An active site in native lipase from Y. lipolytica corresponds to the catalytic triad present in lipases and includes Ser 162, Asp 230 and His 289 (Fickers et al., 2011; Wierckx et al., 2018). Cutinase from Fusarium solani f. sp. pisi (CUT_FS) has a pH range of 3–11 and optimum of pH 6–9. The CUT_FS temperature range is 20–70 °C with optima at 30 and 40 °C (Baker et al., 2012; Chen et al., 2008). Cutinase from Trichoderma reesei (CUT_TR) has two pH optima (4.0 and 7.3). This enzyme is active between pH 3 and 8 and loses stability at a temperature over 60 °C (Roussel et al., 2014). Moreover, both cutinases have a lid covering the active site of the enzyme (Chen et al., 2013; Roussel et al., 2014) and the presence of a biosurfactant in the reaction environment is necessary. Native lipase Lip2 has an optimal hydrolysis temperature of 37 °C and pH 7.0 with functional pH range of 5.5–9.0 (Yu et al., 2007). The enzyme exhibits stability between 25 °C and 55 °C and pH profile 3.5–9. Since Y. lipolytica is known as a biosurfactant producer, and it produces many native lipases (Miller and Alper, 2019), these features can overcome this limitation in the efficient action of both cutinase enzymes.

In this study, we compared the capability of the obtained strains to hydrolyze plastics using PCL as a model compound. To increase their enzymatic abilities, we overexpressed the enzymes simultaneously for the synergic activity. We focused on two different cutinases with various pH optimal activity and native lipase. Finally, we demonstrated that simple co-expression of cutinase from F. solani and lipase from Y. lipolytica results in rapid and efficient biodegradation of aliphatic polyester during a short-term fermentation process.

2. Materials and methods

2.1. Microorganisms and media

In this study we used strains derived from the wild type *Yarrowia lipolytica* A101 (Wojtatowicz and Rymowicz, 1991).

Strains used in this study are shown in Table 1. For *Escherichia coli* strains LB medium was used. YPD (1% yeast extract, 1% peptone, 2% glucose) was used as inoculum medium for *Y. lipolytica* strains. For RNA isolation strains were inoculated in YNB minimal medium with 2% glucose. Shake flask experiments for esterase activity assay were provided in 0.3 L Erlenmeyer flasks in YPD medium at 28 °C at 200 RPM. Spot tests were conducted on YNB minimal medium with 0.1% ε-caprolactone. Emulsification of ε-caprolactone was performed according to the protocol described previously (Kitamoto et al., 2011). pH of agar plates was adjusted before sterilization using phosphate buffer solutions appropriately at pH 4.0, pH 7.0 and pH 9.0.

2.2. Cloning and transformation protocols

Restriction enzymes used in this study were purchased from FastDigest Thermo Scientific (USA). Digestion was conducted according to standard protocols. Target genes were amplified using Phusion high fidelity DNA polymerase (Thermo Fisher Scientific) by PCR reaction. T4 DNA Ligase (Thermo Fisher Scientific) was used in ligation reactions conducted at room temperature for 10 min. Constructed plasmids were isolated using the Plasmid Mini Kit (A&A Biotechnology, Poland). The Genomic Mini AX Yeast Spit kit (A&A Biotechnology, Poland) was used for genomic DNA (gDNA) isolation from yeast. Transformants of *E. coli* and Y. lipolytica were checked using standard PCR reaction with Taq polymerase (PCR Mix, A&A Biotechnology, Poland).

Table 1
Strains and plasmids used in this study.

Strain	Genotype	Source
Escherichia coli		
DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA 96 deoR nupG purB20 ϕ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17 ($r_K^- m_K^+$), λ^-	(Hanahan and Glover, 1985)
pAD Lip2	plasmid carrying the UAS1B16- TEF promoter, YALI0A20350g and XPR2 terminator, amp^r	Janek et al. (2020)
pAD Cut_FS	Plasmid carrying UAS1B16-TEF promoter, codon optimized cutinase gene from Fusarium solani f.sp. pisi fungi and XPR2 terminator, amp^r	This study
pAD Cut_TR	Plasmid carrying $UAS1B_{16}$ –TEF promoter, codon optimized cutinase gene from Trichoderma reesei and XPR2, amp^r	This study
Yarrowia lipolytica		
A101	Wild type	Wojtatowicz and Rymowicz (1991)
AJD	MATA, A101: ura 3-302	(Mirończuk et al., 2015)
AJD ΔAΔX (AJD2)	AJD, ΔAXP (YALI0B05654g), ΔXPR2 (YALI0F31889g)	Janek et al. (2020)
AJD2 pAD Lip2	Overexpression YALI0A20350g in AJD2	Janek et al. (2020)
AJD2 pAD Cut_FS	Overexpression of cutinase from *F. solani f. sp. pisi*	This study
AJD2 pAD Cut_TR	Overexpression of cutinase from *T. reesei*	This study
AJD2 pAD Lip2/ Cut_FS	Overexpression of Lip2(YALI0A20350g) and cutinase from *F. solani f.sp. pisi*	This study
AJD2 pAD Lip2/ Cut_TR	Overexpression of Lip2(YALI0A20350g) and cutinase from *T. reesei*	This study

2.3. Strain construction

All plasmids used in this study were prepared based on the pAD vector (Mirończuk et al., 2017). Cutinase genes (cutinase from F. solani f. sp. pisi 741 bp and from T. reesei 722 bp) were codon-optimized for *Y. lipolytica* and fused to the XPR2 signal sequence from this yeast for protein secretion. Genes were cloned into the pAD vector using SgSI and *Nhe*I sites, resulting in pAD_CUT_FS and pAD_CUT_TR plasmids. All plasmids containing overexpression cassettes were sequenced (Genomed, Poland). The newly obtained vectors were digested with *Mss*I, resulting in linear expression cassettes with Y. lipolytica rDNA, which avoided bacterial DNA integration in the *Y. lipolytica* genome. *Y. lipolytica* transformation was described previously (Mirończuk et al., 2019). Strains containing two genes, cutinase and lipase, were restored before the second transformation using the Cre-Lox recombinase system (Fickers et al., 2003).

2.4. RNA isolation and qRT-PCR

For RNA isolation shake flask cultures were inoculated in YNB medium with 2% glucose. After 24 h of incubation at 28 °C at 200 RPM samples were collected and centrifuged for 5 min at 15 000 RPM. RNA extraction was done using a Total RNA Mini Plus kit (A&A Biotechnology, Poland). Isolated RNA was treated with DNase (RNase-free) (Thermo Scientific) enzyme under the conditions recommended by the manufacturer. Biochrom WPA Biowave II (Biochrom Ltd., UK) was used for RNA quantity measurement. Isolated RNA samples were stored in a freezer at −80 °C. For cDNA synthesis the Maxima First Strand cDNA Synthesis kit (Thermo Fisher Scientific) was used. The DyNAmo Flash SYBR Green qPCR Kit was used for qRT-PCR analysis. The sequences of primers used in qRT-PCR are shown in Supplementary Data. The experiment was carried out in CFX Connect, and data analysis was performed in CFX Maestro Software (BIO-RAD, USA).

2.5. Growth curves

The growth of the strains was tested using Spark Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). For inoculum preparation strains were grown for 24 h in YPD medium. Cultures were centrifuged at 5000 RPM for 3 min and washed twice with sterile Milli-Q water. The experiment was performed in 96-well plates in 200 μl of YPD medium with cell culture standardized to OD_{600} 0.1. Each strain was grown in three repetitions at 28 °C under orbital constant agitation. Optical density of cultures was measured at 600 nm every 30 min for 48 h.

2.6. Spot-test experiments

Emulsion of PCL for the spot test was prepared as described before (Urbanek et al., 2017). Briefly, agar plates congaing 0.1% PCL were prepared using YNB medium with pH standardized to 4.0, 7.0 and 9.0 and mixed with the appropriate volume of 0.5% PCL emulsion. Inoculum cultures were prepared in YPD medium in 0.3 L Erlenmeyer shake flasks at 28 °C at continuous agitation 200 RPM and washed twice in sterile Milli-Q water before OD standardization to 0.1.5 μl of the standardized cell suspension was applied in triplicate on each plate and incubated for 72 h at 28 °C.

2.7. Enzyme assay

Cultures for esterase enzyme activity assay were conducted in 0.3 L shake flasks in YPD medium at 28 °C at 200 RPM. The overnight cultures were grown in YPD medium in flasks. Then inocula were spun down and washed twice with sterile Milli-Q water. Initial OD_{600} for each strain was standardized to 1.0. The experiment was carried out in YPD medium. Samples (500 μl) were collected after every 24 h for 3 days, then were centrifuged at 15 000 RPM for 10 min. Supernatants were transferred to a new Eppendorf tube and frozen at −20 °C for the next experiment.

The enzymatic activity was determined using esterase activity assay with 100 mM p-nitrophenyl acetate dissolved in DMSO. As a reaction environment, 50 mM phosphate buffer at pH 7.0 and pH 9.0 was used. 5 μl of each supernatant was used in each reaction. Buffer and p-nitrophenyl acetate were mixed directly before measurement. The total reaction volume was 150 μl. Absorbance was measured at 37 °C at $\lambda = 405$ nm for 10 min using a BioTek Microtiter Plate Reader. The enzymatic activity was determined by measuring the amount of p-nitrophenol produced at 37 °C for 10 min. The calculated enzyme activity is presented in units. One unit hydrolyzes 1 μmol of substrate per minute under specific reaction conditions.

2.8. Shake flask experiment

The ability of the constructed strains to degrade PCL films was tested in a shake-flask experiment in YPD medium adjusted to pH 4.0, pH 7.0 and pH 9.0 in a 0.3 L Erlenmeyer flask. PCL films were prepared by dissolving poly-ε-caprolactone granules in dichloromethane and poured into glass petri dishes to evaporate the solvent. Dried PCL films were cut into 0.5 g fragments and sterilized by washing in ethanol and subjecting to UV radiation for 30 min. The inoculation cultures were performed by incubation of overnight culture in YPD broth at 28 °C and 200 RPM. Cells were centrifuged at 3000 RPM for 3 min and washed twice with sterile water. Initial OD_{600} for each experimental flask was standardized to 1.0. An amount of 0.5 g of PCL film was added to each flask containing 30 ml YPD medium with the proper inoculant. A control experiment for plastic was carried out with the same procedure but without inoculation. For HPLC analysis samples were collected every 24 h. Samples were collected every 24 h for 72 h. PCL films were collected after 144 h of culture, rinsed with distilled water, dried and weighed. Weight loss percentage analysis was calculated by comparing the weight of PCL film before and after cultivation (Urbanek et al., 2017).

2.9. Analysis of PCL film hydrolysis product

Samples taken during the shake flask experiment were analyzed with the UltiMate 3000 UHPLC System (Thermo Fisher Scientific, Waltham, USA) using a HyperRez CarbH + column with a UV (λ = 210 nm) (Dionex, Sunnyvale, USA) and RI detector (Shodex, Ogimachi, Japan). 25 mM trifluoroacetic acid (TFA) was used as an eluent. Column temperature was set to 65 °C and the flow rate was 0.6 ml min^{-1}. As a standard 1% ε-caprolactone dissolved in methanol was used. The data were analyzed in the Chromeleon Chromatography Data System (Thermo Fisher Scientific, Waltham, USA).

2.10. Phylogenetic analysis

The sequences of domains belonging to the α/β-hydrolase superfamily identified in cutinases and lipases were analyzed. Domain coordinates were taken from Conserved Domain Database (CDD; Lu et al., 2020). The exact position of the domain within PETase has not been established; therefore the entire enzyme sequence was analyzed. Sequences were aligned in COBALT (Papadopoulos and Agarwala, 2007) available at the NCBI webpage (https://www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi). CLC sequence viewer v.8 (QIAGEN Digital Insights) was used for graphical visualization of the alignment and for construction of the phylogenetic tree using the neighbor-joining (NJ) method with Jukes-Cantor protein distance measure after 1000 bootstrap replications (Lu et al., 2020; Papadopoulos and Agarwala, 2007).

3. Results

3.1. Sequence analysis and cloning of cutinases from F. solani f.sp. pisi and T. reesei and lipase from Y. lipolytica

Y. lipolytica is known as an unconventional yeast able to use a hydrophobic carbon source such as alkanes or fatty acids. Its natural ability to produce biosurfactants increases the hydrophilicity of organic compounds, enabling this yeast to assimilate hydrophobic substrates. This feature combined with resistance to high osmotic pressure and low pH makes *Y. lipolytica* a very good host that can be used in the degradation of plastics.

First, to avoid heterologous proteins degradation, we overexpressed the native lipase Lip2 (YALI0A20350g) and two heterological cutinases from *F. solani f. sp. pisi* (K02640.1) and *T. reesei* (XM006964471.1) in a *Y. lipolytica* strain with deleted acid extracellular protease AXP (YALI0B05654g) and alkaline extracellular protease XPR2 (YALI0F31889g) (AJD ΔAΔX) (Janek et al., 2020), hereafter named AJD2. Based on previously published amino acid sequences of various hydrolases, we analyzed the sequences of three tested enzymes, since it is important to identify the differences and commonalities for each enzyme. We assessed the relationship between the amino acid sequence domains of enzymes from the abhydrolase superfamily in 14 species of microorganisms. As seen in Fig. 1A, Lip2 from *Y. lipolytica* (AFH77825) showed the closest phylogenetic homology to PETase from *Ideonella sakaiensis* (6EQD_A) (Yoshida et al., 2016), whereas cutinase from *T. reesei* (ETS02914) showed high homology (53.49% identity) with cutinase from Monilinia fructicola (AAZ95012) (Wang et al., 2002). Cutinase from *F. solani* (AAA33334) is closely related (55.33% identity) to the cutinase from Humicola insolens (4OYY_A) (Kold et al., 2014; Soliday et al., 1984). Cutinases from *F. solani* and *H. insolens* have a common ancestral protein with cutinase from *Colletotrichum gloeosporioides* (AAL38030) (protein homology 48.89% and 58.51% respectively) (Chen et al., 2007; Farah Diba et al., 2001). AAA33334 is homologues with cutinases from *Aspergillus oryzae* (3GBS_A) (50.50% identity) and *Aspergillus nidulans* (ABF50887) (48.74% identity) (Bauer et al., 2006; Zhiqiang et al., 2009). These proteins are found in three clades in which two monophyletic groups can be distinguished, and due to the relatively strong differentiation of homologues, we will be able to assess the efficiency of hydrolysis of ester bonds present in PCL. As seen in Fig. 1b, a highly conserved region has been found in all 14 compared amino acid sequences. It consists of 5 amino acids, the arrangement of which can be represented by G-X1-S-X2-G. In the case of cutinases compared in this alignment, the X1 position is occupied by tyrosine and X2 by glutamine. In lipases we can detect the presence of histidine in X1 and leucine in X2 position. In PETase tryptophan can be found in X1 and methionine in the X2 position. Considering the similarities found in the nucleophilic elbow between the cutinases and the lipase selected for this research, and the

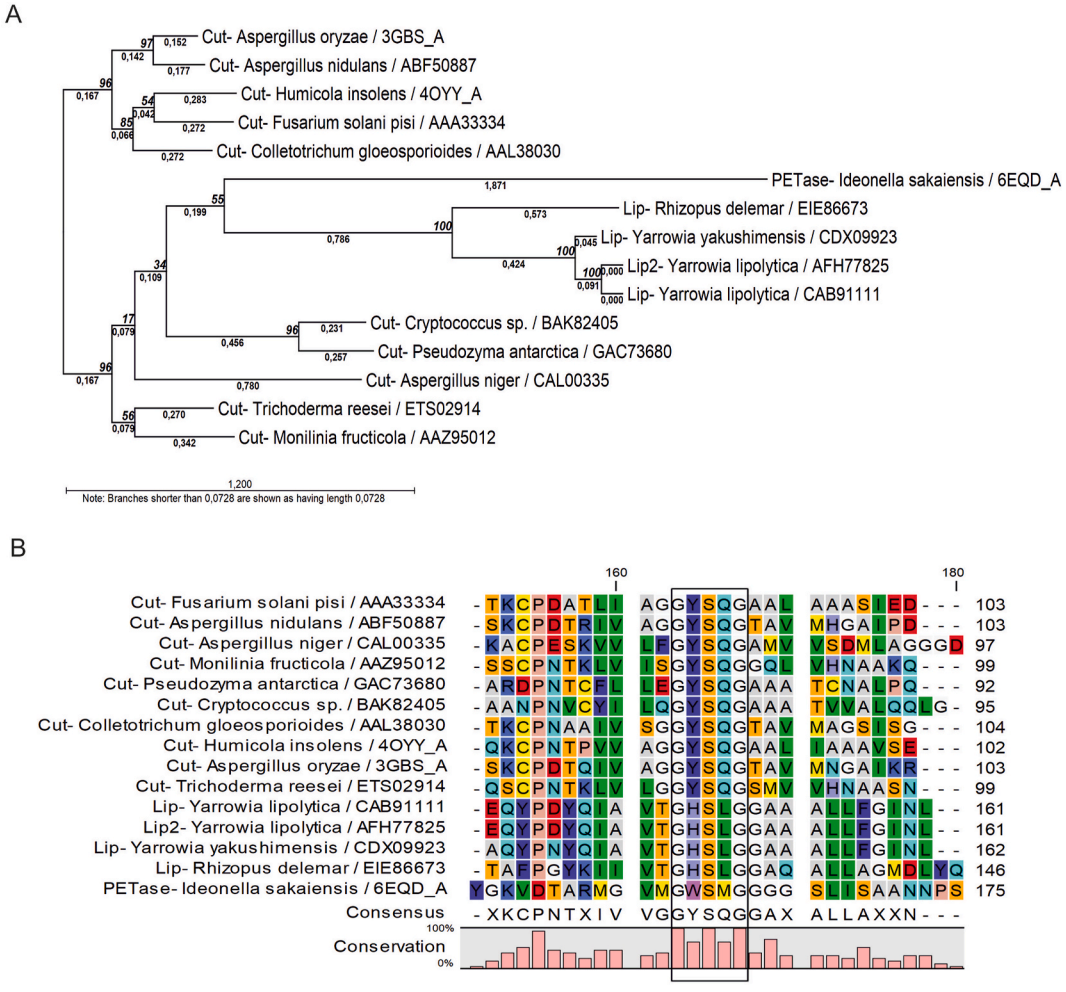

Fig. 1. Phylogenetic relationship between amino acid sequences of abhydrolase superfamily domains found in different cutinases (Cut) and lipases (Lip) of selected yeasts and molds. Accession numbers are given after the slash. The phylogram was constructed with the neighbor-joining (NJ) method and verified by 1000 bootstrap replications. The bootstrap percentage is presented on each node. B) Fragment of the COBALT alignment (Papadopoulos and Agarwala, 2007) visualized with CLC sequence viewer (QIAGEN Digital Insights) of domains belonging to abhydrolase superfamily detected on amino acid sequences of different cutinases (Cut), lipases (Lip) and PETase of the selected microorganisms.

enzyme PETase, they are highly likely to be able to hydrolyze ester bonds in non-biodegradable materials such as polyethylene terephthalate (PET).

3.2. Expression levels of CUT_TR, CUT_FS and Lip2 and growth of the engineered Y. lipolytica

To prove functional overexpression of Lip2 and expression of CUT_TR and CUT_FS of the selected genes we evaluated their expression levels using the qRT-PCR method. For this reason, RNA from the engineered strains was isolated, and *Y. lipolytica* AJD was used as a control. All genes used in this study were cloned under the UAS1B$_{16}$-TEF hybrid promoter with the highest activity observed after 24 h of growth (Blazeck et al., 2013). Fig. 2A shows that all overexpressed genes present elevated expression. The expression level of native lipase in the control strain is low and it is the result of the native promoter's activity. Relative gene expression for the overexpressed lipase was at a similar level in all three engineered strains. The results show that CUT_FS was expressed more highly in single gene overexpression than in co-expression with Lip2. CUT_TR was expressed at a similar level both in single gene overexpression and in co-expression with Lip2. The expression level of Lip2 in the constructed strains is significantly higher than in the control strain which suggests that the gene is functionally overexpressed and the amount of lipase produced by AJD pAD Lip2 strain will be greater than the control strain.

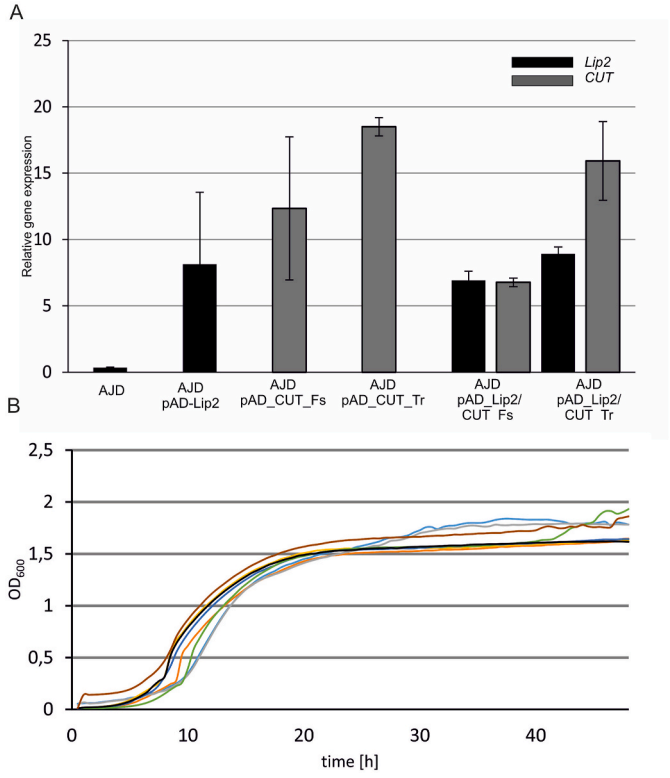

Fig. 2. Relative quantification of RNA transcript in the control strain (AJD) and the engineered *Y. lipolytica* strains overexpressing cutinase from *F. solani pisi* (CUT_FS), cutinase from *T. reesei* (CUT_TR) and/or native *Y. lipolytica* lipase Lip2 using RT-PCR (A). As a reference gene actin was used. All tested strains were grown in YNB medium supplemented with glucose. All samples were analyzed in triplicate and the standard errors were estimated using CFX Maestro Software (BIO RAD, USA). Growth curves (B) of *Y. lipolytica* strains on YPD medium. Experiments were performed at 28 °C with continuous shaking using Spark Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). A101 – dark blue, AJD – grey, AJD2 – orange, AJD2 pAD Lip2 – yellow, AJD2 pAD CUT_FS – light blue, ADJ2 pAD CUT_TR – green, AJD2 pAD Lip_CUT_FS – black, AJD2 pAD Lip_CUT_TRred.

Because metabolic engineering of the microorganisms might delay growth and reduce the ability to produce the target protein (Ko et al., 2020), we tested the difference in growth between the genetically modified strains and control strains (AJD, AJD2). The strains were grown in YPD medium for 48 h. As shown in Fig. 2B, all of the strains showed an extended lag phase. However, all of the engineered strains have a similar growth profile with the exponential phase occurring until 18 h of culture. The highest growth was observed after 20 h. That result confirms that there are no differences in growth level between the genetically modified strains and the control strains.

3.3. Degradation of polymers by the engineered Y. lipolytica strains

The previous experiments showed that the engineered strains are capable of functional overexpression of the cutinases and lipase; moreover, their growth rate remained unchanged in comparison to the control strain. Thus, the next step in our research was evaluation of their degradation abilities. To test them we used emulsified poly-ε-caprolactone (PCL) as a model for aliphatic polyester with ester bonds in its structure, similar to polyethylene terephthalate (PET).

Previous research showed that the optimal temperature and pH are important factors for efficient hydrolysis on plastic polymers (Eberl et al., 2009; Marten et al., 2003; Pischedda et al., 2019). For this reason, here we used two cutinases with various optimal pH values. Therefore, in this study a wide range of pH conditions were used (4.0, 7.0, 9.0) to confirm the ability of *Y. lipolytica* to produce active enzyme at various pH levels. The test was performed at 28 °C, corresponding to the optimal temperature for *Y. lipolytica* growth.

The ability of the obtained strains to degrade PCL was evaluated by clear zone analysis in a spot test experiment. The results are shown in Fig. 3. All the tested strains were spotted onto YNB supplemented with 0.1% PCL. pH was standardized to 4.0, 7.0 and 9.0 according to the previously described optimum pH range for cutinases. In agreement with the assumption, the clear zone method confirmed that strains overexpressing CUT_TR and Lip2_CUT_TR show the highest hydrolytic activity at pH 4.0 (Fig. 3). On the plate with pH 4, other tested strains did not show any activity. At pH 7.0, the biggest clear zones were observed for the strains AJD2 pAD-CUT_FS and AJD2 pAD_Lip2_Cut_FS. Compared to the control strain a significant difference is also visible for AJD2 pAD_CUT_TR and AJD2 pAD_Lip2_CUT_TR strains, but the latter with co-expression of lipase and cutinase from T. reesei has lower enzymatic activity than the strain with single gene expression of CUT_TR. The double-gene overexpressing strain AJD2 pAD_-Lip2_CUT_TR creates slightly bigger clear zones than AJD2 pAD_Lip2. Clear zones created by the strain AJD2 pAD_CUT_FS both at pH 7.0 and pH 9.0 are similar in size. It was found that the strain with single lipase gene overexpression (AJD2 pAD_Lip2) created the biggest zone at pH 9.0. In addition, the clear zone created by the control strain (AJD) was biggest at pH 9.0 compared to the other pH conditions used in this study, which corresponds with presence of the native lipase gene. According to the AJD2 pAD_CUT_TR feature, the clear zones are also noticeable at pH 9.0, but they are smaller than those obtained under other conditions. As shown in Fig. 3, native lipase showed the highest hydrolytic activity towards PCL emulsion at pH 9.0. Our study provides considerable evidence that *Y. lipolytica* can efficiently produce enzymes that show activity at various pH levels already after 72 h of incubation.

3.4. Esterase activity of strains overexpressing CUT_TR, CUT_FS and Lip2

Previous results showed that overexpression of the cutinases and lipase in *Y. lipolytica* results in PCL degradation. Thus, to test the enzymatic activity of the engineered strains, we measured the level of their esterase activity by monitoring the hydrolysis of p-nitrophenyl acetate.

The test was carried out only at pH 7 and 9. Here, we did not perform tests at pH 4.0, due to the fact that nitrophenol is colorless below pH 5.0 and the obtained results would not be reliable at this pH. The results

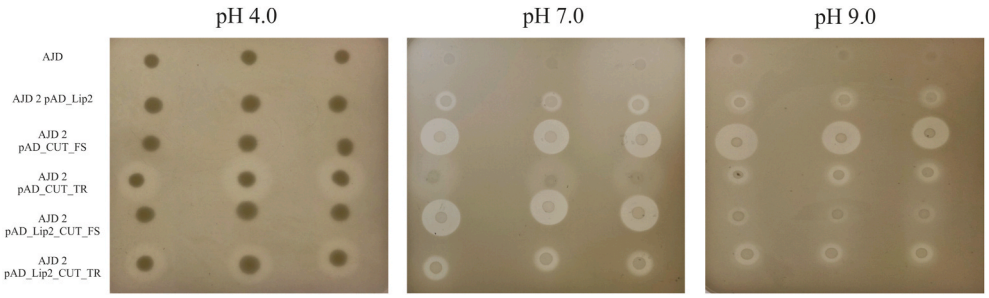

Fig. 3. Degradation of PCL emulsion on the YNB agar plates by the tested *Y. lipolytica* strains at pH 4.0, 7.0 and 9.0. The images were taken after 72 h of incubation at 28 °C.

obtained in the esterase activity assay for the engineered strains at pH 7.0 (Fig. 4A) showed that strains containing cutinase from F. solani f. sp. pisi has the highest enzymatic activity, and reaches 4.58 U ml^{-1} for both AJD2 pAD_CUT_FS and AJD2 pAD_Lip2_CUT_TR strains. This result indicates that lipase overexpression does not have a significant impact on the esterase activity at pH 7.0 after 72 h of culture. Esterase activity at neutral pH is at similar level in AJD2 pAD_Lip2, AJD2 pAD_CUT_TR and AJD2 pAD_Lip2_CUT_TR strains and reaches approximately 0.3 U ml^{-1}. Interestingly, it was observed that strains overexpressing CUT_TR and Lip2 had significantly higher esterase activity measured at pH 7.0 after 24 h of cultivation (data not shown). AJD2 pAD_Lip2, AJD2 pAD_CUT_TR and AJD2 pAD_Lip2_CUT_TR obtained accordingly 3.66 U ml^{-1}, 0.51 U ml^{-1} and 3.80 U ml^{-1}, respectively. We did not observe a similar phenomenon for the strains overexpressing CUT_FS. The decrease in lipase and CUT_TR activity during the cultivation time may be related to the low stability of these enzymes under these conditions. In the control strain (AJD), the native lipase activity is noticeable after 48 h of cultivation (data not shown).

The results obtained for each of the modified strains at pH 9.0 were significantly higher than those obtained for the control strain. The highest activity for AJD2 pAD_CUT_FS and AJD2 pAD_Lip2_CUT_FS strains at pH 9.0 reached 63 U ml^{-1} and 63.5 U ml^{-1} respectively (Fig. 4B). Strains overexpressing cutinase from T. reesei achieved the activity at the level of 20 U ml^{-1} and 13.7 U ml^{-1}, for a single gene and co-expression with Lip2, respectively. This experiment is further evidence that heterologous proteins secreted by *Y. lipolytica* retain their functionality at different pH levels and show high enzymatic activity.

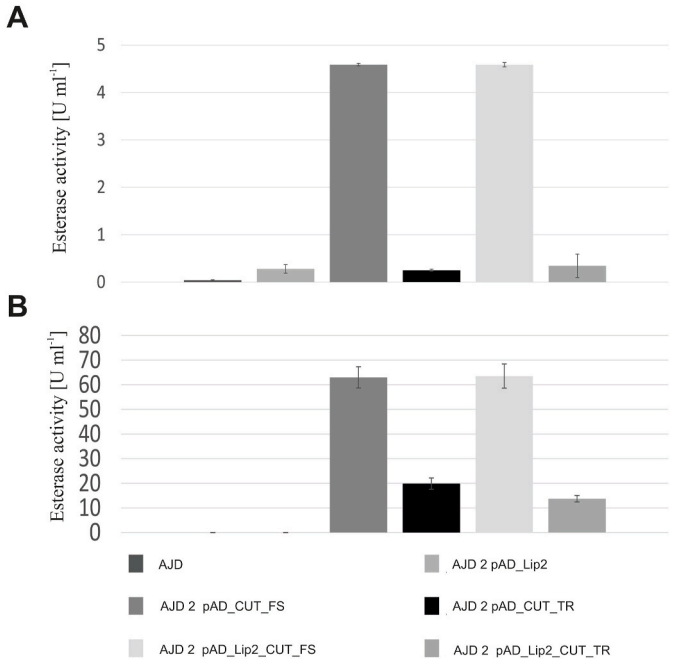

Fig. 4. Esterase activity of the tested *Y. lipolytica* strains at pH 7.0 (A) and 9.0 (B) after 72 h of cultivation in YPD medium.

3.5. Shake-flask experiments with the engineered Y. lipolytica strains

To investigate the capability of the engineered strain to degrade plastic, we performed shake-flask experiments, with PCL films, to estimate the percentage weight loss during cultivation and to detect the products of PCL hydrolysis such as of ε-caprolactone and 6-hydroxyhexanoic acid. Cultures were grown for 144 h with in YPD medium with addition of 0.5 g of PCL film. The percentage weight loss was calculated based on the average weights of the PCL film from three biological replicates. The results were calculated against a control sample incubated under the same conditions but without the presence of microorganisms. The highest percentage weight loss was observed for strains AJD2 pAD_CUT_FS and AJD2 pAD_Lip_CUT_FS (above 90%) (Supplementeary Material, Fig. S2). Strains with CUT_TR and Lip_CUT_TR overexpression also decompose PCL films, but the percentage weight loss is much lower at 9.8% and 15.8% respectively. Interestingly, in this experiment, the synergistic effect of cutinases and Lip2 is clearly visible. In the case of co-expression of these two genes, the percentage weight loss shown by AJD2 pAD_Lip2_CUT_TR was higher than AJD2 pAD_CUT_TR. The visual comparison of the PCL films after incubation with the CUT_FS and Lip2_CUT_FS overexpressing strains in our study, very clearly shows the difference in the appearance of PCL films, and the results are in agreement with the obtained weight loss (Graphical Abstract).

However, the percentage weight loss of PCL films during incubation with the engineered *Y. lipolytica* strains, may lead to misconceptions about the effectiveness of the method used to biodegrade plastic. A major source of uncertainty in this method is the fact that polymers release micro- and nano-plastics during decomposition processes (Wei et al., 2020). In this study we investigated ε-caprolactone release during PCL biodegradation by high performance liquid chromatography analysis (HPLC). As shown in Fig. 5, amounts of ε-caprolactone vary over the cultivation period. Results after 24 h of cultivation can be found in Fig. 5A. The highest amount of ε-caprolactone was observed in AJD2 pAD_CUT_FS and AJD2 pAD_Lip2_CUT_FS samples, 5.55 g L^{-1} and 3.32 g L^{-1}, respectively. For the strain overexpressing CUT_TR, only 0.06 g L^{-1} of this compound was noted in the supernatant. The CUT_TR and Lip2 co-expressing strain caused the release of a slightly larger amount of ε-caprolactone compared to the single overexpression strain, which was 0.09 g L^{-1}. Interestingly, a greater amount of ε-caprolactone was found in the supernatant of the AJD control strain than in the strain overexpressing native lipase. Fig. 5B presents amounts of ε-caprolactone released after 48 h of cultivation. It is clearly visible that the sample derived from the AJD2 pAD_Lip2_CUT_FS strain contained the highest amount of ε-caprolactone, which reached almost 8 g L^{-1}. In the supernatant from the AJD2 pAD CUT_FS strain a similar amount of this released ε-caprolactone as after 24 h of cultivation was observed. A

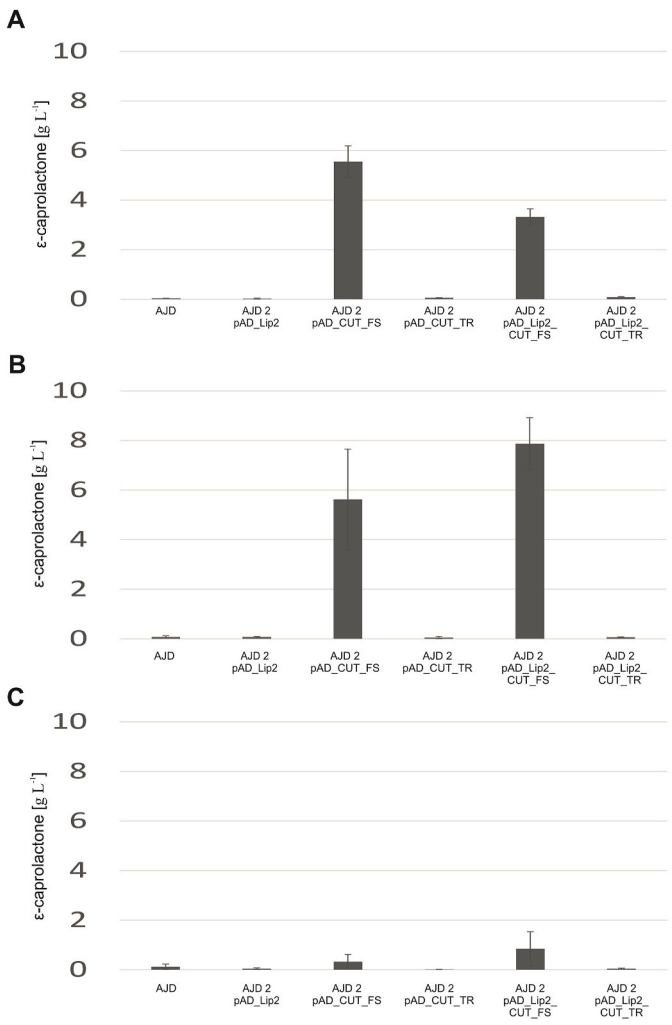

Fig. 5. Hydrolysis of PCL films during shake-flask experiments at 28 °C. Concentration of ε-caprolactone monomers in the supernatant. The samples (in triplicate) were taken after 24 (A), 48 (B) and 72 (C) of incubation. Error bars represent standard deviations.

similar amount of ε-caprolactone was also found in the samples from the AJD2 pAD_Lip2, AJD2 pAD_CUT_TR, AJD2 pAD_Lip2_CUT_TR strains and the control strain. The amount of ε-caprolactone obtained for AJD and AJD2 pAD_Lip2 was twice as high compared with samples collected after 24 h of cultivation. After 72 h of cultivation (Fig. 5C), a large decrease in the amount of ε-caprolactone in the samples from AJD2 pAD_CUT_FS and AJD2 pAD_Lip2_CUT_FS was observed; probably further hydrolysis of ε-caprolactone occurred. In the strain with co-expression of native lipase and cutinase from F. solani the amount of ε-caprolactone found in the supernatant was 2.5 times higher than in the AJD2 pAD_CUT_FS sample. The amount of ε-caprolactone measured after 72 h for the AJD2 pAD_CUT_TR and AJD2 pAD_Lip2_CUT_TR strains compared to the samples collected after 24 and 48 h of culture was also lower. The results obtained for the control strain indicated that 0.12 g of L^{-1} ε-caprolactone was in the supernatant after 72 h, which was the highest amount recorded for the AJD strain throughout the entire cultivation period. The results obtained in this analysis clearly show that the AJD2 pAD_CUT_FS and AJD2 pAD_Lip2_CUT_FS strains degrade PCL most efficiently. After 48 h of cultivation, the synergistic effect of native lipase and cutinase from Fusarium solani, resulting in a higher biodegradation efficiency of PCL by the co-expressing strain Lip2 and CUT_FS, is clearly visible. Moreover, the control strain, AJD, also degraded PCL, releasing the most ε-caprolactone (0.12 g L^{-1}) after 72 h of cultivation. This amount is slightly greater than that of the AJD2 pAD_Lip2, AJD2 pAD_CUT_TR and AJD2 pAD_Lip2_CUT_TR strains, which did not reach a similar value at any of the examined samples.

In summary, our experiment showed that the engineered strains of *Y. lipolytica* during 72 h of cultivation are capable degrading PCL films into monomers.

4. Discussion

Growing amount of plastic wastes forces the scientist to find the solution of this global issue. Several microorganisms have been reported to be capable of plastic degradation, but their natural capabilities are limited. Up to now, an evidence for a significant effect of enzymes from the hydrolase class of cutinases, lipases and PETase has already been reported (Carniel et al., 2017; Herrero Acero et al., 2011; Kawai et al., 2019; Shi et al., 2020; Yoshida et al., 2016). For this reason we functionally overexpressed three different hydrolases, cutinase from *F. solani f. sp. pisi*, cutinase from *T. reesei* and native lipase Lip2, in the yeast *Y. lipolytica*. To prove, we tested the gene expression in the engineered strains, interestingly, we observed the differences between expressed genes. However, the obtained results are related to the level of transcription factors in a cell, and it might result in lower gene expression in strains containing two overexpressed genes under the same hybrid-promoter. A similar phenomenon was observed in all strains with co-expression used in this study. Previous studies have shown that functional co-expression of two genes can be performed and the level of gene expression of the cloned genes may differ between strains with single gene overexpression (Dobrowolski and Mirończuk, 2020; Tai and Stephanopoulos, 2013).

As mentioned before the optimal temperature and pH are important factors for efficient hydrolysis on plastic polymers. To test this, we performed esterase activity assays at various pH to measure the activity of the expressed enzymes. The results obtained in our experiments are consistent with previous results documenting optimal pH for cutinases from F. solani f. sp. pisi (7.0 and 9.0) and T. reesei (4.0 and 7.0) (Maruyama et al., 2003; Poulsen et al., 2005; Roussel et al., 2014). The level of lipase activity in the control strain (AJD) is consistent with the previous studies. So far, documented activity of the native *Y. lipolytica* lipase after 72 h at pH 7.0 was below 0.5 U ml^{-1} with the cultivation carried out in the bench fermenter and olive oil supplementation (Alonso et al., 2005). Lipase activity determined by Yu et al. on the purified Lip2 from *Y. lipolytica* indicated pH 8.0 as the most optimal one. At pH 7.0 and 9.0 lipase activity was respectively 50% and 85% lower than at pH 8.0 (Yu et al., 2007).

Next, to determine the level of polyester degradation we performed a shake flask experiments with the PCL films. The material was sterilized by washing in ethanol and subjected to UV irradiation for 30 min. These methods might have an influence on the structure of the plastic material, but with a short exposure of PCL to these factors, no negative effects on the morphology have been found in previous studies (Horakova et al., 2020). Poly-ε-caprolactone was used in our experiment as a model substrate, due to the fact that hydrolysis of this compound may indicate a potential use in the degradation of more complex compounds such as polyethylene terephthalate (PET). It is known that this substrate was used as a model polymer in other studies (Almeida et al., 2019; Danso et al., 2018; Nyyssölä et al., 2013). The highest degree of degradation was observed for strains AJD2 pAD CUT_FS and AJD2 pAD Lip_CUT_FS. In contrast to other studies (Shi et al., 2020), we did not note any significant weight loss change after PCL films' exposure for 144 h in AJD2 pAD_Lip2 culture. However, we observed a decrease in lipase activity after 24 h of cultivation, which may cause this effect. The investigation by Shi et al. was carried out with purified lipase enzyme with established activity up to 45 U ml^{-1}. Also cutinase from F. solani f. sp. pisi in 45 U ml^{-1} enzyme solution resulted in weight loss of around 85% after 72 h of incubation, but the published photographs do not indicate such significant degradation of the PCL films by this enzyme (Shi et al., 2020).

A major source of uncertainty during measurement the weight loss of

polyester's film is the fact, that polymers might be fragmented into micro- and nano-plastics during decomposition processes. To avoid this inaccuracy, we measured the level of the released ε-caprolactone during fermentation process. This compound was detected in all tested samples, however the highest level was observed for strain expressing CUT_FS. Before, the presence of PCL degradation products using both the natural abilities of microorganisms and incubation with purified enzymes was described. As reported by Hoshino and Isono in 2002, complete biodegradation of PCL by lipase F hydrolase from Rhizopus oryzae into monomers is possible after 100 days of incubation with the purified enzyme, and it was shown that dimers were the main decomposition products of PCL (Hoshino and Isono, 2002). Presence of ε-caprolactone was detected in HPLC analysis by Oda et al., in 1995 in a PCL degradation experiment with PCL depolymerase produced by the filamentous fungus P. lilacinus D218. However, that experiment was carried out for 1 h with purified enzyme solution. In our study we cultivated the engineered strains with polyesters films, what significantly reduce time and the cost of the process.

In summary, our experiment showed that the engineered strains of *Y. lipolytica* overexpressing CUT_FS and Lip2 during 72 h of cultivation are capable degrading PCL films into monomers.

5. Conclusion

The obtained results showed that co-expression of the heterologous cutinase and native lipase in yeast *Y. lipolytica* results in efficient polyester degradation at 28 °C. The secreted enzymes remain stable, active with capability to degrade of 0.5g PCL during 144 h of cultivation. The obtained results are a good starting point for further optimization of hydrolysis of non-biodegradable polyesters such as PET.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2021.105232.

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Efficient biodegradation of aliphatic polyester by genetically engineered strains of the yeast *Yarrowia lipolytica*

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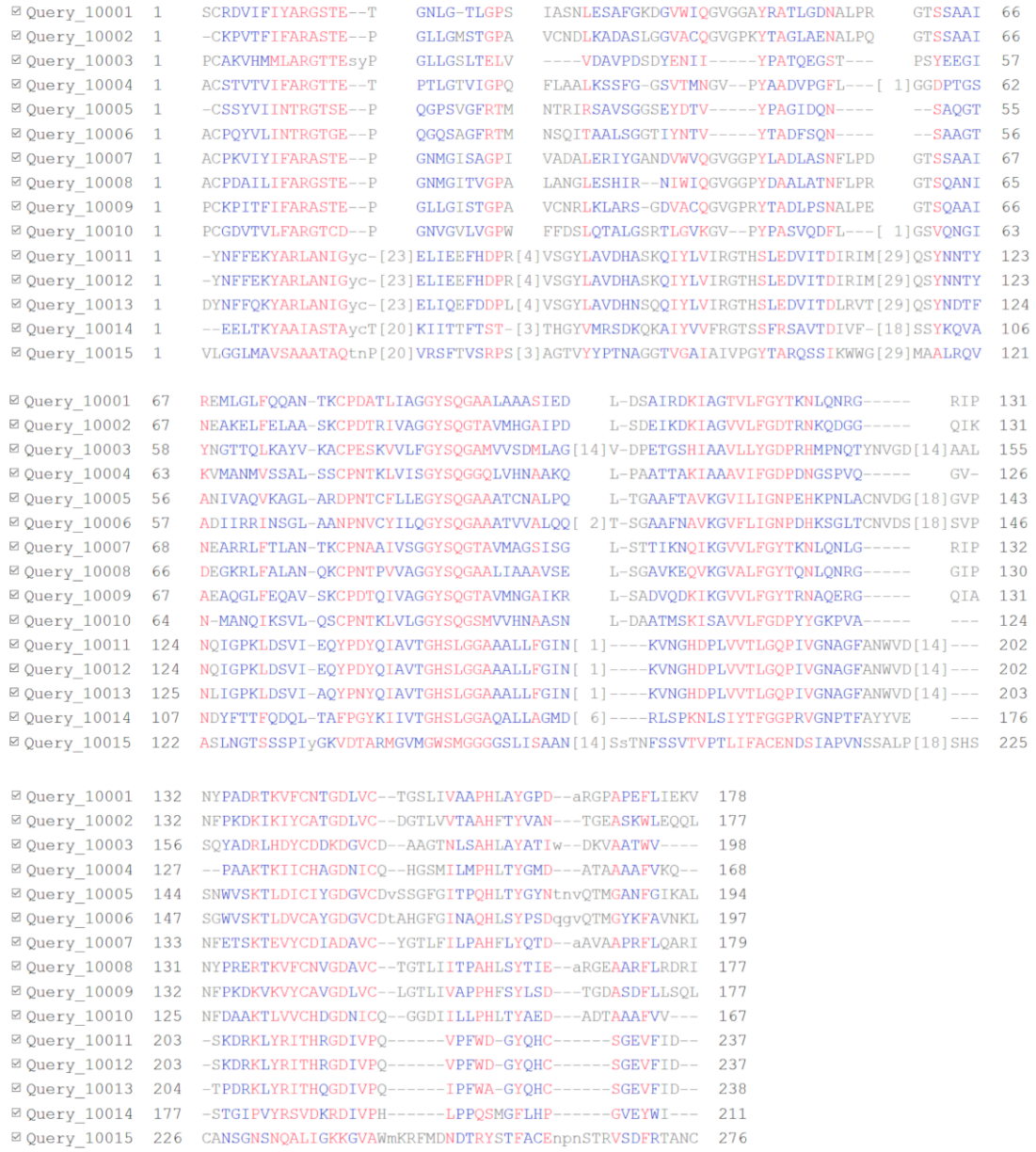

Figure S1. The alignment of amino acid sequences of domains belonging to abhydrolase superfamily detected in different cutinases, lipases and of full sequence of PETase chain A.

Red and blue colors indicate highly and less conserved positions, respectively. Grey amino acids are not conserved. Sequence accesion numbers are given after slash.1. Cut- *Fusarium solani pisi* / gb / AAA33334, 2. Cut- *Aspergillus nidulans* / gb / ABF50887, 3. Cut- *Aspergillus niger* / emb / CAL00335, 4. Cut- *Monilinia fructicola* / gb /AAZ95012, 5. Cut- *Moesziomyces (Pseudozyma) antarctica* / dbj/ GAC73680, 6. Cut- *Cryptococcus sp.* / dbj/ BAK82405, 7. Cut- *Colletotrichum gloeosporioides* / gb / AAL38030, 8. Cut- *Humicola insolens* / pdb/ 4OYY_A, 9. Cut- *Aspergillus oryzae* / pdb /3GBS_A, 10. Cut- *Trichoderma reesei* / gb / ETS02914, 11. Lip- *Yarrowia lipolytica* / emb /CAB91111, 12. Lip2- *Yarrowia lipolytica* / gb / AFH77825, 13. Lip- *Yarrowia yakushimensis* / emb /CDX09923, 14. Lip- *Rhizopus delemar* / gb / EIE86673, 15. PETase- Ideonella sakaiensis /6EQD_A

Table S1. RT-PCR primers sequences used in this study.

Name	Sequence 5'-3'
qACT_F	GAGTCACCGGTATCGTTC
qACT_R	GCGGAGTTGGTGAAAGAG
qLip2_F	TCCTTCTGAGGCCGCAGTTC
qLip2_R	TTGCGAGTCGGGCGTACTTC
qCut_FS_F	TCGCCCTCACCACTTTACTG
qCut_FS_R	AATCGTCTCGGGTGGTTCTG
qCut_TR_F	ACCGCCTTCACTATTCTGAC
qCut_TR_R	GTGTCACCGATGGGCATCAC

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Metabolic engineering of *Yarrowia lipolytica* for poly(ethylene terephthalate) degradation

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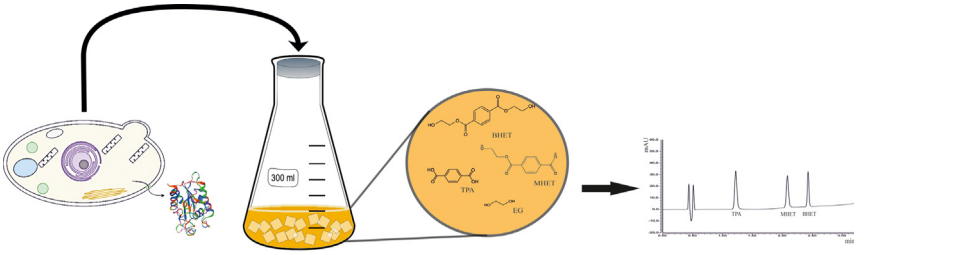
HIGHLIGHTS

- Overexpression of cutinase in *Y. lipolytica* results in PET films degradation.
- The process does not require the PET pretreatment.
- *Y. lipolytica* is able to degrade PET at 28 °C during the fermentation.
- Differences in the ratio of released degradation products were observed.
- The PET film samples after cultivation possess numerous damages on the surface.

GRAPHICAL ABSTRACT



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ABSTRACT

Polyethylene terephthalate (PET) is the most widely used plastic, whose global production scale causes serious problems due to it being highly non-biodegradable. The present work provides a novel approach to plastic degradation studies, which involves direct degradation of PET in the culture of a modified *Y. lipolytica* yeast strain extracellularly producing cutinase from *Fusarium solani*. In this study, we successfully accomplished a scale-up of the degradation process in culture, which is promising from the perspective of wider application of the developed method in the future. Additionally, we tested the effect of various supplements, which may increase the PET degradation efficiency in the culture of the *Y. lipolytica* pAD CUT_FS strain. The ability of PET decomposition was verified by the amount of the released degradation products, such as terephthalic acid (TPA) and mono-(2-hydroxyethyl)-terephthalic acid (MHET), during cultivation. We observed that the quantities of TPA and MHET released during the PET degradation process were increasing daily, and were 1.51 gL^{-1} and 0.45 gL^{-1}, respectively after 240 h of the bioreactor fermentation. Analysis of the PET film by electron microscopy indicated that there was abundant damage on the surface of the material. This study also demonstrated that the engineered *Y. lipolytica* strain is able to degrade PET at 28 °C during fermentation. The results obtained in this study using amorphous PET powder provide a wide range of possibilities for application of the cutinase-secreting strain of *Y. lipolytica* on the more difficult to degrade highly crystalline PET films, PET bottles and PET melts.

1. Introduction

The development and improvement of plastic production technology in the last century has significantly affected industrial progress and improved the quality of life, and due to its mechanical and thermal properties, its range of applications is very extensive. One of the world's most commonly used plastic polymers is polyethylene terephthalate (PET). Due to the widespread use of this material, both the production and the waste generation are constantly increasing. Recycling processes help combat the accumulation of plastic waste; however, in 2018, only about 9% of PET trash was recycled (Garcia et al., 2021). Over time the accumulation of plastic debris in coastal areas, seas, and oceans has become a serious threat to the health and lives of wildlife (Jambeck et al., 2015). PET was classified as a non-

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biodegradable polymer, for which estimated calculations for PET half-life parameters based on the SSDR method (specific substrate degradation rate) have shown that it can take over 2500 years (Chamas et al., 2020). Additionally, the process of plastic degradation in the environment is influenced by PET properties such as the crystallinity and the environmental conditions (landfill, soil, marine) under which the degradation occurs (Li et al., 2016; Pirzadeh et al., 2007). Other factors affecting the degradation time of PET are the high hydrophobicity of this polymer and the high molecular weight, impeding the transfer of the molecule into the cell (Moharir and Kumar, 2019; Oberbeckmann and Labrenz, 2020). Moreover, the effective enzymatic decomposition of PET occurs at a temperature close to the glass transition temperature, which in the case of this polymer is 75–80 °C due to the increased mobility of the polymer chain (Kawai et al., 2014; Priya et al., 2021). The process carried out at this temperature requires an energy supply and only a few known enzymes are stable under this condition for a long time. Degradation of PET using hydrolytic enzymes leads to the release of hydrolysis products such as BHET (bis(2-hydroxyethyl) terephthalate), MHET (mono-(2-hydroxyethyl) terephthalic acid), TPA (terephthalic acid) and EG (ethylene glycol) (Mohanan et al., 2020). The exact scheme along with an example chromatogram of the tested compounds is shown in Fig. 1. One possibility to solve the problem of plastic waste accumulation is its biodegradation by microorganisms since some of them are able to secrete hydrolytic enzymes that break the ester bond present in PET. For this reason, many studies have focused on the identification and application of the enzymes from the esterase subclass in the biodegradation of this polyester. In several studies the authors have proposed the use of cutinases from *Thermobifida fusca*, *Fusarium oxysporum* and *Fusarium solani pisi* as promising enzymes in the context of PET degradation (Furukawa et al., 2019; Groß et al., 2017; Nimchua et al., 2007). Subsequently, researchers have made attempts to enhance PET degradation by modifying the structure of the cutinase enzyme (Joo et al., 2018; Son et al., 2019; Tournier et al., 2020).

The aim of this study was to investigate the *Y. lipolytica* AJD 2 pAD CUT_FS strain capability to decompose PET directly in the culture by analysing the concentration of the released degradation products of this polymer. Our results indicate that efficient PET degradation is possible without the necessity of conducting the process at temperatures approaching the glass transition temperature. In our work, we also performed a scale-up of the degradation process to evaluate the possible application of the proposed method on a large scale. The obtained results are a good starting point for further study on plastic degradation by the engineered *Y. lipolytica* strains. Furthermore, the ability of this yeast to assimilate atypical carbon sources may offer interesting possibilities of uptake of the degradation products released during cultivation, thus allowing them to continuously grow and simultaneously produce PET degrading enzyme.

Table 1
Strains used in this study.

Strain	Genotype	Source
Yarrowia lipolytica		
AJD 2	MATA, A101, ΔAXP, ΔXPR2	(Janek et al., 2020)
AJD 2 pAD CUT_FS	MATA, A101, ΔAXP, ΔXPR2, overexpression of cutinase from *Fusarium solani f.sp. pisi* (CUT_FS)	(Kosiorowska et al., 2021)

2. Materials and methods

2.1. Microorganisms and media

The strains used in this study AJD 2 (Janek et al., 2020) and AJD 2 pAD CUT_FS are derived from the wild type *Yarrowia lipolytica* A101 strain and are listed in Table 1. The codon optimized cutinase gene fused with the XPR2 signal sequence was cloned into the pAD (Mirończuk et al., 2017) vector, resulting in the construct of the pAD CUT_FS plasmid. Subsequently, the obtained vector was digested using the MssI restriction enzyme and cloned into the *Y. lipolytic*a strain without acidic and alkaline proteases (ΔAXP (YALI0B05654g), ΔXPR2 (YALI0F31889g)), further named as the AJD 2 strain. Detailed cloning, transformation protocols, and strain construction of *Y. lipolytica* AJD 2 pAD CUT_FS were described previously (Kosiorowska et al., 2021).

Media for inoculation and RNA isolation used in our study were YPD (1% yeast extract, 2% peptone, 2% glucose). YP medium with 5% glucose

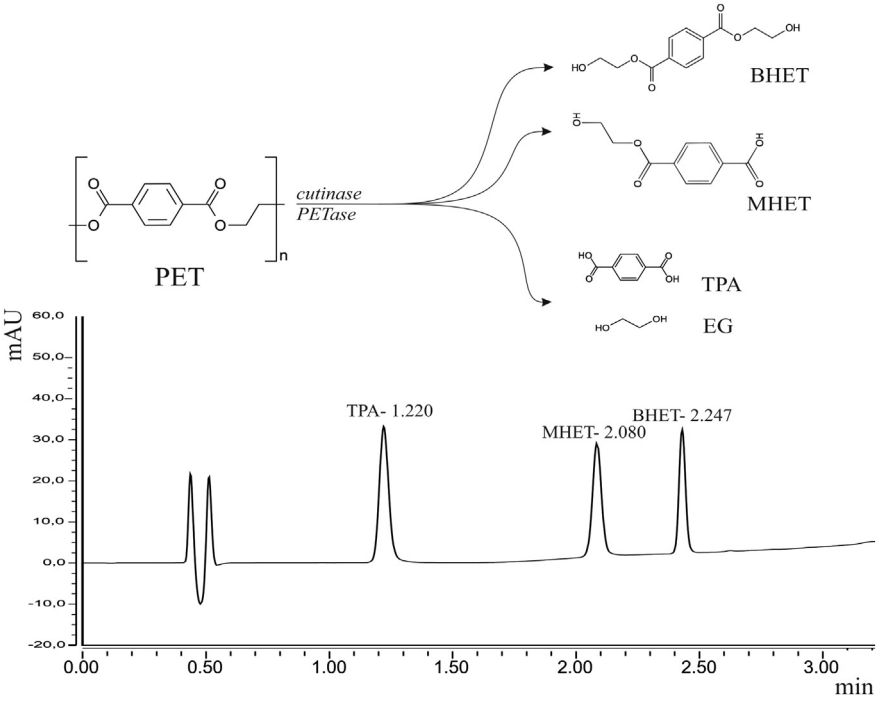

Fig. 1. Enzymatic degradation of poly(ethylene terephthalate) (PET) and chromatographic distribution of its degradation products: TPA (terephthalic acid), MHET (mono(2-hydroxyethyl) terephthalic acid), and BHET (bis(2-hydroxyethyl) terephthalate) on a C-18 column.

was used for shake cultures conducted in deep well plates (DWP), 0.3 L Erlenmeyer flasks, and bioreactors.

2.2. RNA isolation and qRT-PCR

The cultures for RNA isolation were grown in 10 mL of YPD medium in 100 mL flasks at 28 °C and 200 RPM. Samples for RNA isolation were collected after 24 h of incubation and the isolation of RNA was carried out using the Total RNA Mini Plus Kit in accordance with the manufacturer's protocol (A&A Biotechnology, Poland). For qRT-PCR, isolated RNA samples were treated with DNase (Thermo Scientific) and cDNA synthesis was achieved with the Maxima First Strand cDNA Synthesis kit (Thermo Fisher Scientific). The qRT-PCR analysis was carried out using the DyNAmo Flash SYBR Green qPCR Kit (Thermo Fisher Scientific). Primers qCUT_FS_F (5′-TCGCCCTCACCACTTTACTG-3′) and qCUT_FS R (5′-AATCGTCTCGG GTGGTTCTG-3′) resulted in the product size of 105 bp. The results were normalized to the actin gene amplified with the primers qACT_F (5′-GAGTCACCGGTATCGTTC-3′) and qACT_R (5′- GCGGAGTTGGTGAAAG AG-3′). Samples were analysed in triplicate in CFX Connect and the data analysis was carried out in CFX Maestro Software (BIO-RAD USA).

2.3. Growth curves

The growth analysis of strains was carried out using a Synergy H1 Microtiter Plate Reader (BioTek, USA). The inoculation culture was grown for 24 h in a YPD medium at 28 °C and 200 RPM. Cultures were centrifuged at 3000 RPM for 5 min and washed twice with Milli-Q water. The experiment was conducted in 96-well plates in 200 μL of YP medium with 50 gL^{-1} of glucose. Yeast strains standardized to OD_{600} 0.1 were grown in triplicate at 28 °C with constant orbital agitation with measurement of optical cell density at 600 nm every 30 min for 72 h.

2.4. Deep well plate experiment

The ability of the engineered strain to degrade plastic was evaluated using a deep well plate (DWP) culture. The yeast strain overexpressing cutinase from *F. solani pisi* and the control AJD 2 strain were maintained in 4 mL of YP medium with 5% glucose for 168 h at 28 °C and 200 RPM. Samples were taken daily starting from 72 h of culture. Each culture contained 0.266 g of poly(ethylene terephthalate) (PET) powder (semi-crystalline; 300 μm; GoodFellow ES306031/1, England) previously sterilized for 10 min under a UV lamp added at the culture start time. Deep well plates were also used to screen for supplements that may increase plastic degradation. For this purpose, 50 mM aqueous salt solutions were prepared using hydrated salts: $MnSO_4 \times H_2O$, $ZnSO_4 \times 7H_2O$, $CuSO_4 \times 5H_2O$, $CaCl_2$, $MgSO_4 \times 7H_2O$. The molarity of stocks was calculated concerning the dehydrated salts. Salts were sterilized using 0.22 μm syringe filters (Merck Millipore). Olive oil and canola oil previously sterilized in an autoclave were also used in the study. Experiments on the small laboratory scale were performed in triplicate. Statistical analysis was carried out to determine the significance of the effect of the additives used on the amount of decomposition products released. Analysis was carried out using Student's *t*-test and the significance of the difference between the mean results obtained in the control culture and the adequate supplemented culture was tested (two-tailed; unequal variance; $*p \leq 0.5$, $**p \leq 0.01$, $***p \leq 0.005$).

2.5. Shake flask and bioreactor studies

Experiments performed to determine the ability of the modified yeast strain to degrade plastic on a larger laboratory scale were conducted in 0.3 L Erlenmeyer flasks and a 5-L stirred-tank reactor (BIOSTAT B-PLUS, Sartorius, Germany). The shake flask experiment was carried out for 240 h of continuous agitation of culture at 200 RPM and 28 °C. For culture growth 30 mL of YP medium containing 50 gL^{-1} of glucose and 2 g of PET powder previously sterilized for 10 min with UV radiation was used. The plastic material was added at the beginning of culture. Samples were collected daily starting from 72 h of culture. The bioreactor studies were conducted in the working volume of 1.0 L YP with 50 gL^{-1} of glucose at 28 °C. Aeration and stirring rates were set at 0.8 vessel volume per minute (vvm) and 700 min^{-1}. The pH of 8.5 was maintained automatically by the addition of 20% sodium hydroxide (NaOH) and 10% hydrochloric acid (HCl). Antifoam solution (BEKCHEM S.C., Poland) was automatically added if necessary. PET powder previously sterilized by 10 min of exposure to UV light was added after 48 h of culture and samples were taken daily starting after 72 h of culture duration. The culture duration was 240 h, which corresponds to 192 h of direct degradation time of PET powder in the culture. Cultures in shake flask and bioreactor experiments were conducted in three biological replicates.

2.6. Analytical methods

Samples taken from small-scale experiments (200 μL), shake flask experiments (500 μL) and bioreactor studies (4 mL) were centrifuged at 10,000 G for 10 min. The supernatants were diluted in 99.9% methanol (Chempur) and centrifuged for 10 min at 10,000 G and 10 °C. A standard solution of terephthalic acid (TPA) (CAS: 100-21-0, Sigma-Aldrich) was prepared by dissolving in dimethyl sulfoxide (DMSO) and mono(2-hydroxyethyl) terephthalic acid (MHET) (CAS: 1137-99-1, AChemBlock) and bis(2-hydroxyethyl) terephthalate (BHET) (CAS: 959-26-2, Sigma) by dissolving in 99.9% methanol. Standard solutions in a final concentration of 10 gL^{-1} were stored in the freezer at −20 °C. The standard curve was prepared based on the standard solutions by appropriate dilution of the analytes in 99.9% methanol. Quantitative and qualitative analyses were performed using Ultra Performance Liquid Chromatography (UPLC) with the UltiMate 3000 UHPLC System (Thermo Fisher Scientific, Waltham) using a C18 type column (Hypersil Gold 100 × 2.1 mm, 3 μm) with a universal guard column (Hypersil Gold 10 × 2.1 mm, 3 μm). The two mobile phases used for gradient UPLC elution were (A) acetonitrile with 0.1% trifluoroacetic acid (v/v) and (B) H_2O with 0.1% trifluoroacetic acid (v/v). The gradient separation program used in this study is shown in Table S1. The flow rate was 0.8 $mLmin^{-1}$, the volume injection was 1 μL and the column temperature was 45 °C. Detection of the analytes was done by a UV detector (Dionex, Sunnyvale, USA) at 243 ± 2 nm. The identity of the analytes was additionally confirmed by absorption spectrum analysis in the range of 190–500 nm. The developed method allows the complete separation of analytes within 7 min at a maximum pressure of 250 bar. An example chromatogram and the retention time of the standard mixture containing 10 mgL^{-1} of each of the analytes TPA, MHET, and BHET are shown in Fig. 1. Data analysis was performed using Chromeleon 7.1.

2.7. SEM

To determine the surface morphology changes during PET degradation in the culture of the modified *Y. lipolytica* yeast strain, a shake flask experiment was performed. Yeast cultures were incubated in 50 mL of YPD with a glucose content of 5% for 4 weeks at 28 °C and 200 RPM. A 0.25 mm thick transparent amorphous PET film (GoodFellow ES301445/7, England) was used for the experiments. PET film was previously cut into 1 cm × 1 cm pieces and sterilized for 10 min by UV irradiation.

To verify the influence of the microorganism's activity on the polymer plastic surface, a scanning electron microscope was utilized. The investigation was conducted in three trials ascribed as AJD 2 and AJD 2 pAD CUT_FS under several magnifications (500× and 4000×). No special pre-investigation treatment procedure was applied except thin gold layer sputtering, to avoid build-up of excessive electric load. Sputtering conditions were as follows: current 40 mA, sputtering time: 60 s (Cressington, Sputter Coater 108). SEM micrographs were obtained using a VEGA Tescan 3 microscope and dedicated software.

3. Results and discussion

3.1. Quantification of cutinase gene expression level and growth of the engineered Y. lipolytica strain

In our previous study, we obtained a yeast strain capable of functional overexpression of cutinase from *F. solani pisi*. The *Y. lipolytica* AJD 2 pAD CUT_FS derived in that research can successfully degrade poly-ε-caprolactone (PCL) during shake culture studies (Kosiorowska et al., 2021). Here, we focused on investigating the ability of the engineered *Y. lipolytica* strain to degrade the poly(ethylene terephthalate) (PET) polyester. The relative cutinase expression level in the engineered strain AJD 2 pAD CUT_FS was tested and shown in Supplementary Fig. 1A.

The functional overexpression of cutinase from *F. solani pisi* has been assessed before (Kosiorowska et al., 2021); thus now we have checked the stability of CUT_FS expression in *Y. lipolytica*. To accomplish that, total RNA was isolated and the relative expression of the cutinase gene was tested from *Fusarium solani pisi* in the modified strain and compared to the control strain. Even though the genetic modification may impair the growth of the host, as shown the growth profile of the strain overexpressing cutinase from *Fusarium solani* compared to the control strain (AJD 2) does not differ (Supplementary Fig. 1B). As shown in Sup. Fig. 1B, AJD 2 pAD CUT_FS and AJD 2 strains possess a similar growth profile characterized by an extended lag phase. The exponential growth phase occurred up to 20 h of culture. The growth deceleration associated with depletion of the carbon source exhibited a prolonged late log phase.

3.2. Degradation of poly(ethylene terephthalate) by the engineered Y. lipolytica strain

First, to investigate the ability of the *Y. lipolytica* strain AJD 2 pAD CUT_FS to degrade PET the assays were performed on a small laboratory scale. The experiment was conducted in shake culture with the addition of PET powder; samples were taken every 24 h starting from 72 h of cultivation and the concentration of PET degradation products was determined by UPLC analysis. The amount of PET powder added to the culture was determined by previously conducted studies on optimizing the amount of substrate in the culture for the highest amount of breakdown products being released (data not shown). The results shown in Fig. 2 indicate that degradation products such as terephthalic acid (TPA) and mono(2-hydroxyethyl) terephthalic acid (MHET) are released during yeast culture with PET powder. It was observed that the amount of degradation products increased in time. The highest concentrations of TPA and MHET compounds were observed after 168 h of culture and were 46.96 mgL^{-1} and 79.28 mgL^{-1} respectively. Fig. 2 shows that the largest daily increase in the concentration of hydrolysis products occurred between 144 h and 168 h of culture. These results may be related to the culture pH (shown in supplementary material Fig. S2A), which varied around 4.0 at the first checkpoints (72 h and 96 h), and a significant increase was observed at 126 h of culture. The highest daily increase in the amount of released degradation products was observed on the last two days of sampling when the pH was constant at 8.0, which corresponds to the optimal pH for cutinase activity. In contrast to the other research (Kawai et al., 2019; Xi et al., 2021), bis(2-hydroxyethyl) terephthalate (BHET) and ethylene glycol (EG), which are also products of PET hydrolysis, were not observed. The ability of the control strain to degrade PET was also evaluated; however, no PET degradation products were detected in the supernatants after culture with the *Y. lipolytica* AJD 2 strain (data not shown).

It was found that cutinases do not require the addition of cofactors for an efficient enzymatic reaction (Chen et al., 2013). However, several studies have been published that suggest the positive effects of salt ions on cutinase activity against synthetic polyesters (Kawai et al., 2014; Senga et al., 2020). Due to the ability of lipases to degrade PET as described previously, we also used olive oil and canola oil as supplements to induce extracellular lipase production in *Y. lipolytica* (Fabiszewska et al., 2014). The results of culture supplementation with different concentrations of salt, olive oil, and canola oil are shown in supplementary material Fig. S3. The results obtained in this experiment indicate that the addition of 2.5 mM calcium chloride ($CaCl_2$) had the greatest impact on the amount of TPA released while reducing the amount of MHET released compared to control cultures conducted without supplementation. Also, a larger amount of TPA was observed when a total salt concentration of 2.5 mM $MnSO_4$, 1 mM $CaCl_2$, and 2.5 mM $MgSO_4$ was applied. Interestingly, in the case of 2.5 mM $MnSO_4$ and 2.5 mM $MgSO_4$, the amount of MHET was significantly lower compared to the control.

Next, we increased the scale of the culture to 0.3 L (Erlenmeyer flasks). For this reason, the time of PET powder incubation with the *Y. lipolytica* strain AJD 2 pAD CUT_FS was extended to 10 days (240 h). The samples were collected every 24 h, and the amounts of TPA and MHET were measured. The results are shown in Fig. 3. As expected, we observed a trend of increasing amounts of PET hydrolysis products with the duration of

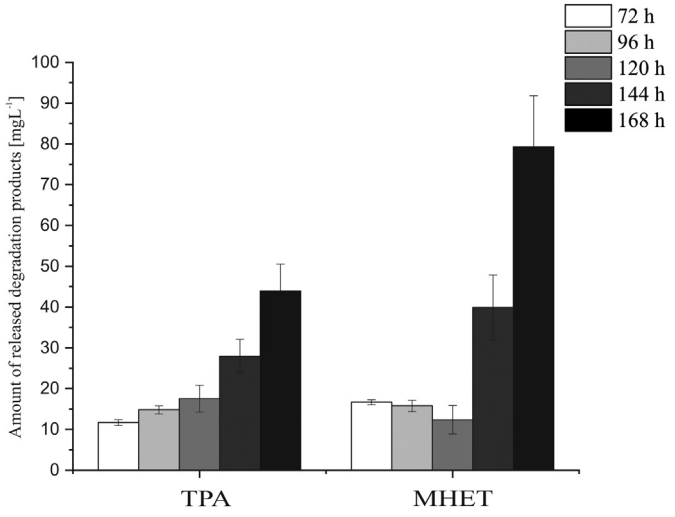

Fig. 2. PET degradation products (TPA and MHET) released during low scale shake AJD 2 pAD CUT_FS culture performed in deep well plates (DWP) in YP medium with 50 gL^{-1} of glucose and 0.26 g of PET powder. The culture was incubated at 28 °C with continuous agitation at 200 RPM. The samples were analysed in triplicate. Standard deviation is shown by error bars.

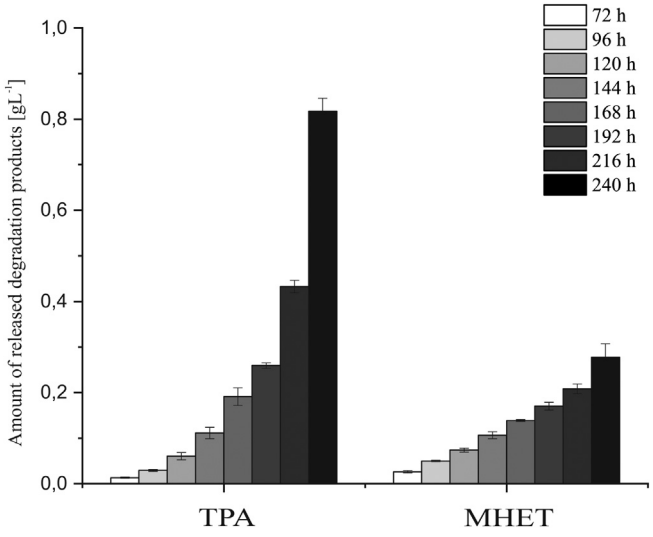

Fig. 3. Amount of PET degradation products (TPA and MHET) released during AJD 2 pAD CUT_FS culture performed in 0.3 L Erlenmeyer flasks in YP medium with 50 gL^{-1} of glucose and 2 g of PET powder. The culture was incubated at 28 °C with continuous agitation at 200 RPM. The samples were analysed in triplicate. Standard deviation is shown by error bars.

culture. The highest levels of PET degradation products were observed on the last day of culture, *i.e.*, after 240 h, and were 0.81 gL^{-1} of TPA and 0.28 gL^{-1} of MHET. Similar to the small-scale culture, the largest daily increase in the amount of TPA and MHET was observed in the supernatant at the last two measurement points, between 216 h and 240 h. The pH in the shake flask cultures remained constant between 8.04 at 72 h of culture and 8.72 at 240 h (Fig. S2B). Similar to the culture performed in deep well plates, no PET degradation products were detected in the supernatant from the shake flask culture of the control strain (AJD 2).

As we successfully increased the scale of PET decomposition to 30 mL of culture, in the following step, we conducted a bioreactor experiment in a working volume of 1 L. Due to the problem caused by foam formation and the high risk of culture contamination connected with maintaining pH constant at the level of 8.5, sterile PET powder was added to the culture at 48 h of fermentation. The pH of the culture at 8.5 was determined based on previous experiments on the enzymatic activity of the supernatant from the strain used in this study (data not shown). Fig. 4 shows the level of the PET degradation products (TPA and MHET) released during the bioreactor study. At the beginning of the bioreactor study, the level of the released degradation products was similar to the small scale (by 168 h of culture, the amount of TPA measured for the flask experiment was 0.19 gL^{-1}, that in the bioreactor culture was 0.14 gL^{-1}, and the MHET concentrations were 0.14 gL^{-1} and 0.09 gL^{-1}, respectively). The obtained results show that at the final measurement point (after 240 h of culture) the amount of TPA was almost double compared to the flask culture and was 1.51 gL^{-1}. The amount of MHET varied during the culture duration; the highest amount of this compound was observed at 216 h of culture and was 0.45 gL^{-1}. Interestingly, at the end of the process, we observed a decreasing amount of MHET in the supernatant. This phenomenon might be related to the further hydrolysis of this compound to TPA, the amount of which significantly increased between 216 and 240 h of culture.

Differences in monomer ratios according to the used process scale and the culture duration were also noted. It was observed that the ratio of TPA to MHET (TPA:MHET) measured on the last day of culture in deep well plates was 0.55:1, for flask cultures 2.95:1, and for bioreactor cultures 4.63:1. This result may be related to the findings of other researchers who have noted that the ratio of released hydrolysis products depends on the concentration of the enzyme acting on the PET (Vertommen et al., 2005). The *Y. lipolytica* yeast used in this study grew more slowly in the cultures conducted in deep well plates due to oxygen depletion, which directly affected the biomass growth and consequently the level of extracellular cutinase production (Mirończuk et al., 2019). The delayed growth of yeast under these conditions can also be seen by analysing pH changes during the culture. There is slower alkalinization of the medium in cultures conducted in deep well plates compared to the cultures grown in 0.3 L Erlenmeyer flasks (Sup. Fig. 2). Additionally, it was noted that the ratio of TPA to MHET was higher in favour of MHET up to 144 h of culture in both shake flasks and the bioreactor, which is also related to the amount of cutinase continuously produced by the yeast. These results indicate that it is more favourable for the PET degradation process to use bioreactor studies, not only due to the greater rate of PET degradation demonstrated by a higher concentration of degradation products but also to direct the hydrolysis process towards the final degradation product, which is TPA. The data obtained are broadly consistent with other studies, which have shown that degradation products such as TPA, MHET, and small amounts of BHET are released during PET degradation by cutinase from *Fusarium solani* (Vertommen et al., 2005). In the data obtained in our experiment a trace level of the BHET compound was detected only in bioreactor cultures after 144 h of culture (data not shown).

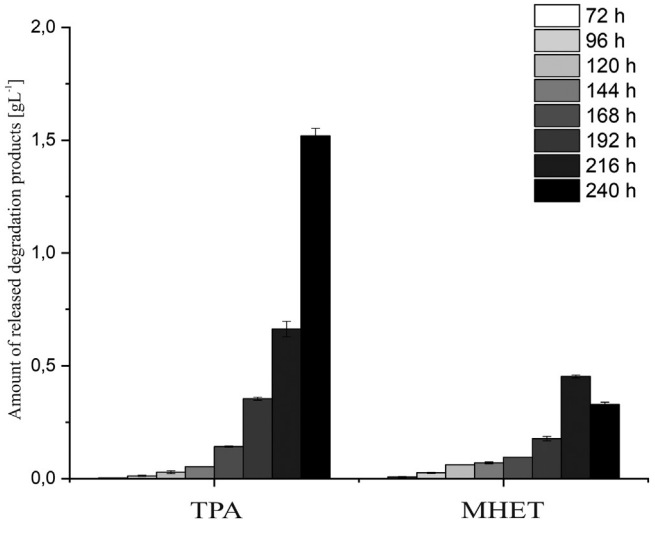

Fig. 4. Amount of PET degradation products (TPA and MHET) measured in the supernatant during AJD 2 pAD CUT_FS culture performed in a bioreactor. The cells grew in 1 L of YP medium with a glucose concentration of 50 gL^{-1} for 240 h. PET powder addition in the amount of 33 g of PET was added after 48 h of culture. The samples were analysed in triplicate. Standard deviation is shown by error bars.

In the last decade, the use of cutinases in plastic degradation studies has attracted much attention from researchers. Several studies have shown that a broad range of cutinases can hydrolyse the ester bonds present in aliphatic polyesters such as poly-ε-caprolactone (PCL) and aromatic polyesters such as PET (Eugenio et al., 2021; Kosiorowska et al., 2021). Recent research has focused on PET degradation using concentrated and purified cutinase enzyme solutions (Eugenio et al., 2021; Gamerith et al., 2017b; Xi et al., 2021). Other researchers investigated the degradation of PET directly with the microorganism possessing PET-hydrolysing activity (Yan et al., 2021) and the effect of culturing microorganisms in consortia on PET and BHET (Roberts et al., 2020).

Many studies have documented the ability of microorganisms to degrade PET, both wild and genetically modified strains (Gamerith et al., 2017b; Roberts et al., 2020). The studies are mainly based on the use of purified enzymes in the PET degradation process. To date, cutinase from *Thermobifida fusca* has been used in partial textile processing to modify PET fibres (Zimmermann and Billig, 2010). Moreover, engineered LCC cutinase from *T. fusca* has been industrially applied to PET degradation (Tournier et al., 2020). Cutinase from *F. solani pisi* has also been functionally expressed in *Pichia pastoris* (Kwon et al., 2009; Ping et al., 2017) or *Bacillus subtilis* (Brockmeier et al., 2006). An interesting experimental demonstration of PET degradation using cutinases from different fungi was conducted by Ronkvist et al. in 2009. The research showed that cutinase from *Humicola isolens* caused 97 ± 3% weight loss of low crystallinity PET film after 96 h of incubation at 70 °C, while incubation of this substrate with cutinase from *Fusarium solani* conducted at 40 °C caused weight loss of 5%. In our research, we did not study the weight loss of plastic due to the release of the micro- and nanoplastics during degradation because this may give a misleading indication of the efficiency of the undertaken process, a problem that has already been highlighted in previous reports (Wei et al., 2020). In a subsequent report, researchers used genetic engineering methods to overexpress thermophilic cutinase (LCC) in the thermophilic anaerobic bacterium *Clostridium thermocellum*. The batch culture of *C. thermocellum* strain overexpressing LCC for 14 days at 60 °C resulted in a 60% decrease in plastic mass, and the total amount of degradation products (TPA and MHET) released during this process was 12 mM (Yan et al., 2020). The use of a higher temperature in the PET decomposition process allows the polymer chains to be more mobile and flexible, allowing better access for the enzymes to the ester bonds (Ronkvist et al., 2009). It is worth noting that these conditions are more suitable regarding energy consumption during the process. Another cutinase-like enzyme, Cut190*, derived from *Saccharomonospora viridis*, an enzyme whose stability is regulated by calcium ion concentration, can successfully perform BHET hydrolysis. The largest amount of MHET released during this study was obtained by adding the 4 mM BHET substrate to the reaction and was about 275 μM MHET. Optimal temperature conditions obtained in the same study indicated that the best temperature for BHET hydrolysis

using Cut190* is 60 °C and the amount determined was about 165 μM of MHET (Hantani et al., 2018). In recent years, there have been several studies comparing the effects of hydrolases that can degrade PET. In one of the reports performed on PET fibres, researchers compared hydrolases from *Fusarium oxysporum* LCH I and *Fusarium solani pisi*. The ability of PET degradation was determined based on the amount of TPA released during the 168 h incubation of plastic material with the 80 U enzyme hydrolysing activity. The amount of TPA released for PET incubation with *F. oxysporum* was over 16 μgmL^{-1} and for the hydrolysis with *F. solani* it was over 9 μgmL^{-1} (Nimchua et al., 2007). Another study investigated the hydrolysis ability of PET films by purified cutinase enzymes from *Thermobifida cellulosilytica* and its mutants. Incubation of cut PET films previously washed in Triton X-100, at 50 °C with 200 μgmL^{-1} of the enzyme was carried out for 2 days. The amount of degradation products released for the unmodified enzymes was approximately 260 μM TPA and 20 μM MHET for Thc_Cut1, for Thc_Cut2 85 μM TPA and 80 μM MHET. For the Arg29Asn_Ala30Val double mutant, the measured amount of TPA was 400 mM and that of MHET was 45 μM. For the triple mutant Arg19Ser_Arg29Asn_Ala30Val, the amount of TPA released was approximately 375 μM and that of MHET was 40 μM (Acero et al., 2013). The other study regarding modification in the protein structure of cutinase to enhance PET degradation was carried out by Wei et al. in 2016. Several mutants of the protein derived from *Thermobifida fusca* KW3 were obtained in this research and its activity was compared to the original TfCut2 enzyme. PET fibres degradation was conducted for 50 h with 50 μg of the purified enzyme at 65 °C (Wei et al., 2016). Successive researchers improved the Thc_Cut1 protein from *Thermobifida cellulosilytica* by fusing it with a polymer binding module from *Alcaligenes faecalis* depolymerase. *E. coli* was used as the host organism to produce the fusion protein and control proteins (Thc_Cut1 and Thc_Cut2). The efficiency of PET degradation was checked by incubating 5 μM of the purified enzyme with PET membranes at 50 °C. The measured concentrations of the degradation products were: for Thc_Cut1 6 μM TPA, Thc_Cut2 2.4 μM TPA and 4.2 μM MHET, and Thc_Cut1_PBM 6.5 μM TPA (Gamerith et al., 2017a). Another cutinase that has been tested for its ability to degrade PET is produced by *Humicola insolens*. The purified HiC enzyme was incubated at 70 °C with post-consumer PET for 96 h and the total concentration of the released TPA, MHET, and BHET compounds was 129 mM (Eugenio et al., 2021). An interesting approach was recently presented by Yan et al., 2021. In this study, *Microbacterium oleivorans* and *Thermobifida fusca* were applied to PET film, and the total concentration of PET degradation products released after 15 h was 90 nM and 217 nM, respectively (Yan et al., 2021). Consecutively, a PE-H hydrolase from *Pseudomonas aestusnigri* and its mutant PE-H Y250S were tested for hydrolytic activity using amorphous PET film and a commercial PET bottle as substrates. The plastic was incubated with 500 nM purified enzyme at 30 °C and the highest measured amount of MHET

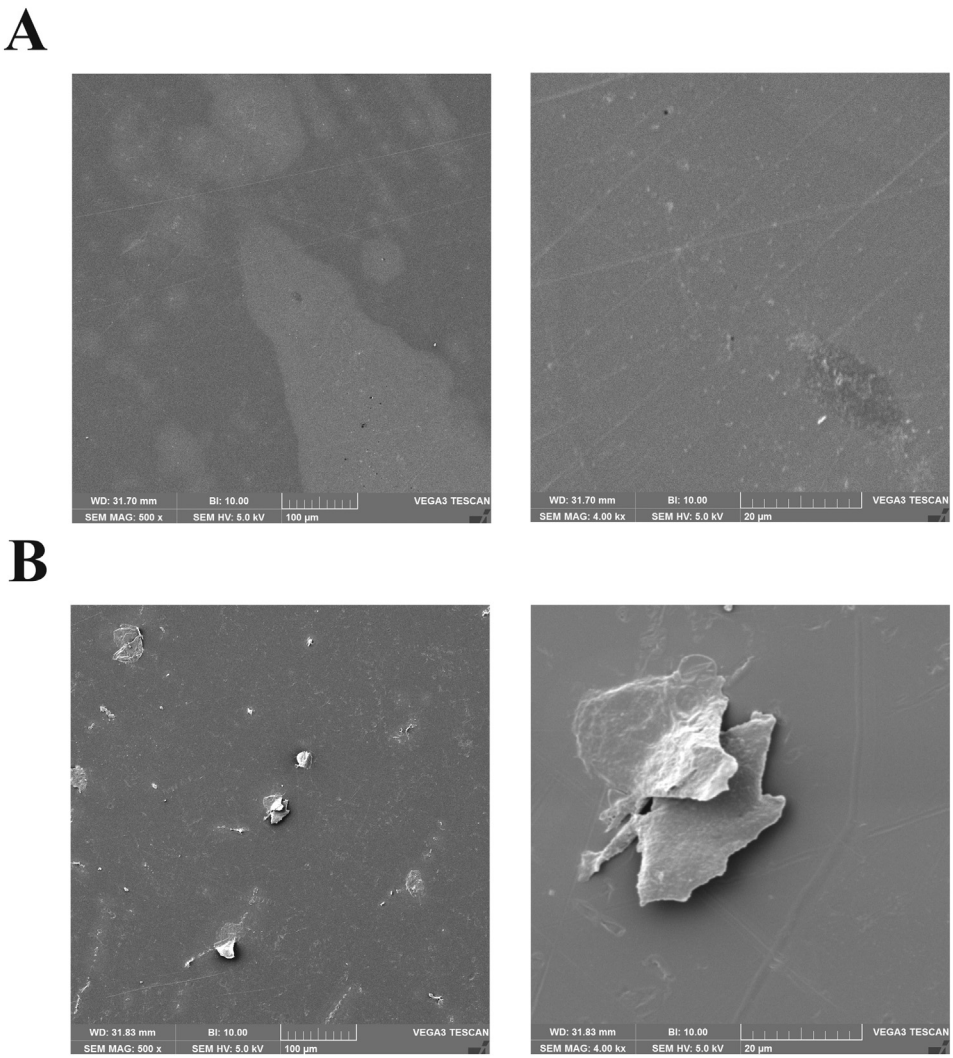

Fig. 5. PET films SEM images after 4 weeks of incubation with AJD 2 (Fig. 6A) and AJD 2 pAD CUT_FS (Fig. 6B) strains in 50 mL of YP medium with 50 gL^{-1} of glucose. Cultivation was performed at 28 °C in 200 RPM in 0.3 L Erlenmeyer flasks. Magnification used to visualize the surface of plastic material was 500× and 4000×. Properties and the scale can be found under the relevant image.

released was 5 mgL^{-1} for PE-H Y250S using PET film as substrate (Bollinger et al., 2020). In a study presented by Xi et al., 2021, *B. subtilis* was used as a host organism for BhrPETas hydrolase and LCC cutinase production. Amorphous PET powder was used as the substrate and the reaction was carried out for 20 h with the purified enzyme at 70 °C. The total amount of PET degradation products released in the BhrPETase-producing strain CBS2ΔhrcA was 6.3 mM and LCC was 5.85 mM (Xi et al., 2021). The quoted results concerning the ability of the aforementioned enzymes to degrade PET are shown in the comparative table (Supplementary Table 1).

Indeed, as can be seen from an analysis of previous works on PET degradation, studies to date have focused on the use of purified proteins under optimum temperature conditions for incubation of the plastic. The efficiency of polymer decomposition was estimated, among other methods, by analysing the concentration of released degradation products. In our work, we have developed the possibility of efficient PET degradation directly in the culture of an organism capable of extracellular production of cutinase. The PET degradation approach used in our work has not been implemented before. Therefore, this study is an important contribution to the extension of research involving the application of fermentation methods to the degradation of plastic waste.

3.3. Surface morphology changes

Next, we analysed the morphology of the surface of PET films cultivated with the engineered strain during shake flask experiments, by scanning electron microscope (SEM). The available literature classifies PET-degrading hydrolases into enzymes that increase the hydrophilicity of the polymer surface with no apparent change in the surface morphology and enzymes that cause significant erosion of the plastic surface (Kawai et al., 2019). Here, we analysed the samples of plastic films after culture with the control strain (AJD 2) and the engineered strain (AJD 2 pAD CUT_FS) under 500× and 4000× magnification. The samples of plastic material for SEM analysis were collected after long-term shake flask culture conducted for 4 weeks at 28 °C and 200 RPM. A visual representation of the changes in surface morphology is shown in Fig. 5. PET film incubated in the control culture (Fig. 5A) does not exhibit significant damage on the surface. Small scratches can be seen on it, which probably occurred during the production processes of the material or the preparation of the films for the experiment. It can be clearly confirmed that incubation of the plastic with the AJD 2 strain does not affect the damage of the polymer. The surface appearance of the PET film after shake flask culture with a modified *Y. lipolytica* strain overexpressing cutinase is entirely different (Fig. 5B). Many abrasions are already visible at a magnification of 500×; the surface of the plastic is damaged, covered with large irregularities, small cracks, and cavities. These results are in agreement with other studies that have shown that during 40-day culture at 30 °C using *Bacillus* and *Pseudomonas* bacteria, numerous defects occur on the surface of PET film (Roberts et al., 2020). Similar observations to ours were described by Markandan and Umamaheswari in 2021. The test performed in their study showed that the PET surface became rough after 30 days of polymer incubation at 37 °C in culture with *Bacillus licheniformis* and *Bacillus cecembensis* (Markandan and Umamaheswari, 2021). The apparent changes in the morphology of PET film obtained in our study, after incubation at low temperature, allows us to apply the presented method for direct degradation of plastic during fermentation, which makes future research on PET degradation very optimistic.

In summary, this study confirms the ability of the applied *Y. lipolytica* modified yeast strain to degrade PET directly in the culture.

4. Conclusion

The main aim of this study was to investigate the ability of AJD 2 pAD CUT_FS to hydrolyse PET during fermentation and to determine the degradation efficiency along with the possibility of adapting the degradation process on a large scale. The study demonstrated that the engineered strain of *Y. lipolytica* is able to degrade PET at 28 °C during the fermentation process. Furthermore, we found that the PET film is also degraded under the applied conditions. Combining the natural capability of *Y. lipolytica* for atypical carbon source utilization and expression of heterologous cutinase results in a low-cost process of plastic degradation. The results obtained in this study allow for future design and optimization of the PET degradation process in fermentation. The present results suggest that the applied novel solution may be an interesting direction for further research on the improvement of the PET degradation process.

CRediT authorship contribution statement

KEK Investigation, conceptualization, data analysis, Writing – original draft.

PB Investigation, Supervision.

AD Investigation, strain construction, Supervision.

KL Investigation, SEM, Visualization.

AMM conceptualization, Project administration, Supervision, Funding acquisition, Writing - review & editing.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154841.

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Metabolic Engineering of *Yarrowia lipolytica* for Poly(ethylene terephthalate) Degradation

Supplementary data

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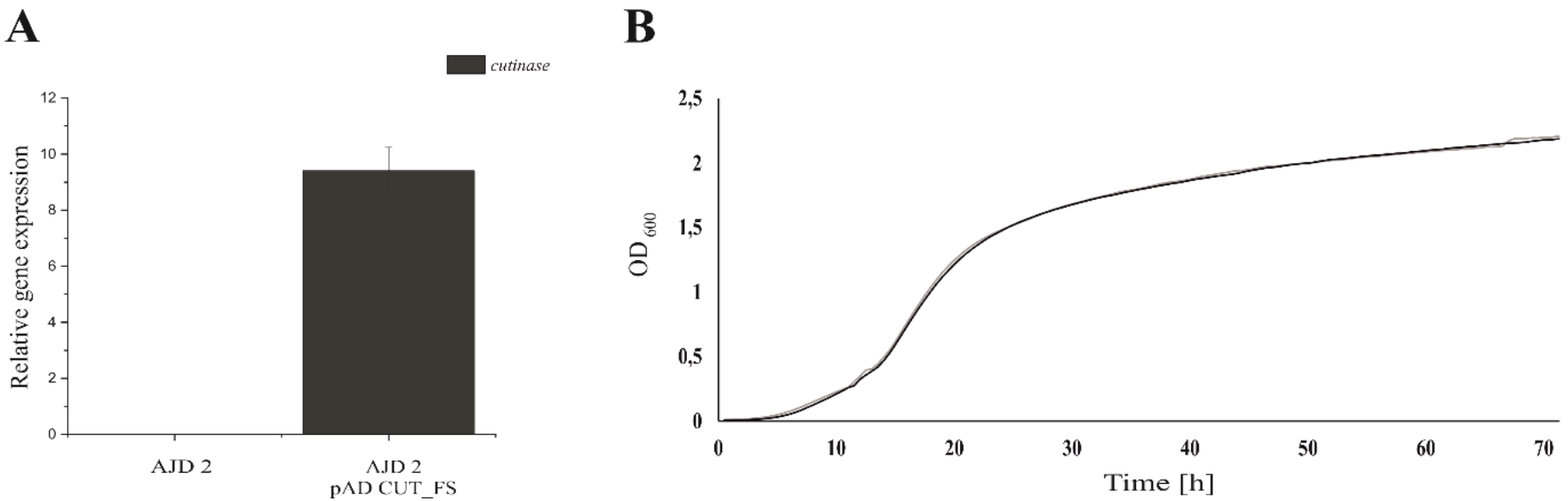
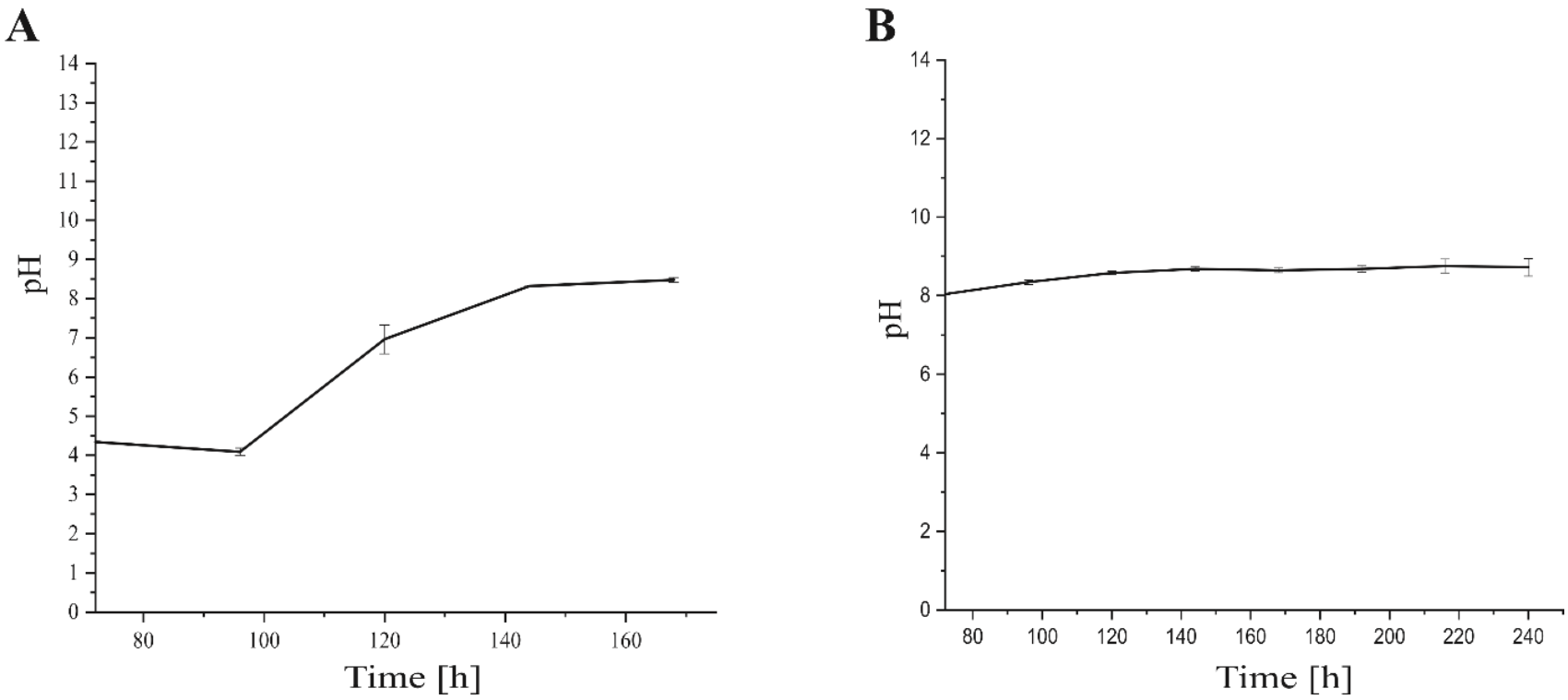


Supplementary Figure 1.

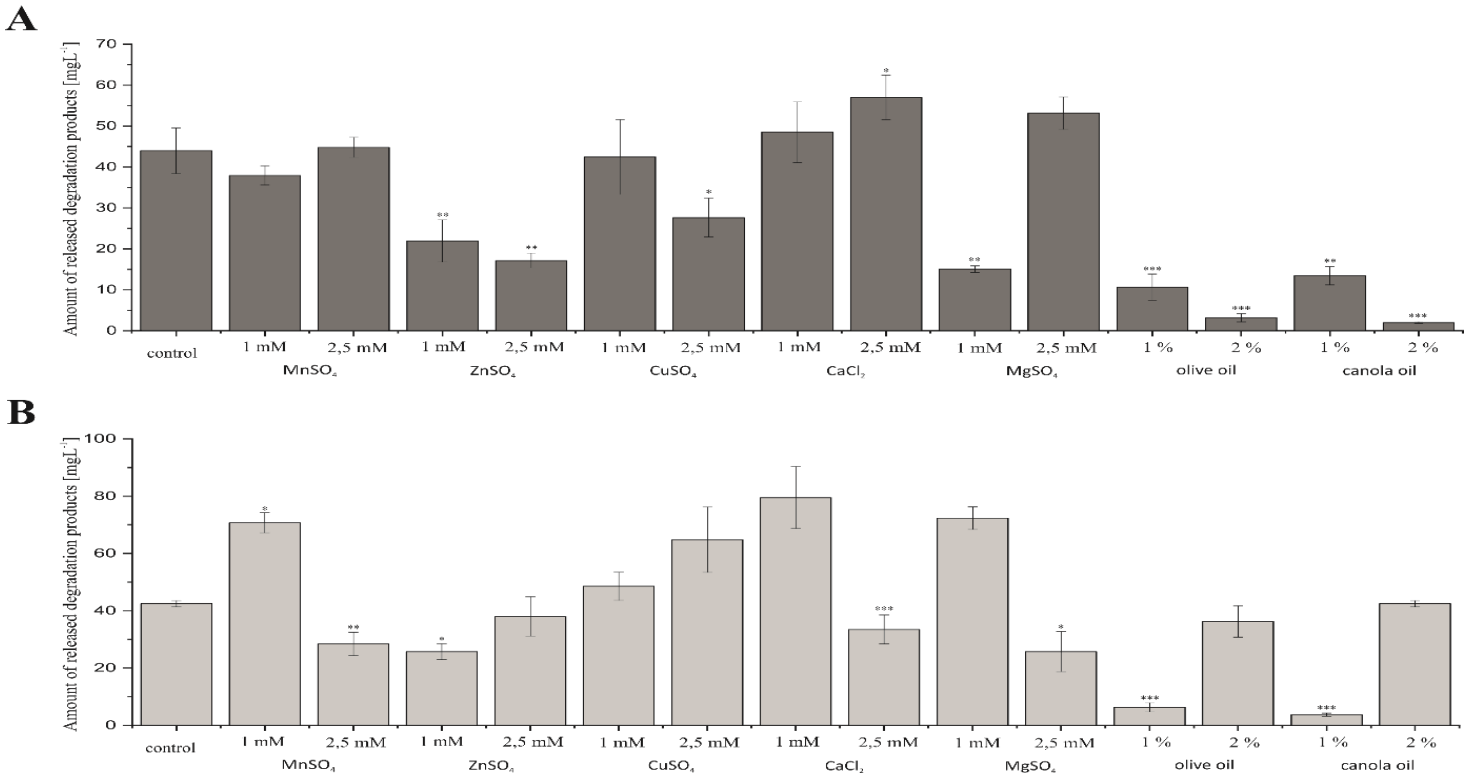
A. Expression level of genes overexpressed in this study. Actin was used as a reference gene. Relative quantification of RNA transcripts using RT-PCR. Samples were analysed in triplicate and standard errors were estimated using BioRad CFX Maestro Software. The error bar is expressed as the standard deviation from the mean.

B. Growth curves of Y. lipolytica stains on and YPD with the glucose content of 5 % medium. Experiments were performed at 28 °C with continuous shaking using BioTek Microtiter Plate Reader for 72 h. Light grey- AJD 2, dark grey- AJD 2 pAD CUT_FS



Supplementary Figure 2.

pH changes during AJD 2 pAD CUT_FS culture performed in DPW (A) and 0.3 L Erlenmeyer shake flasks (B) in YPD medium with 5 % of glucose and 0.266 g (A) or 2g (B) of PET powder. Errors bars are expressed as standard deviation from the mean.



Supplementary Figure 3.

Effect of supplementation of salts, and canola and olive oil in various concentrations, on the amount of PET degradation products released by the AJD 2 pAD CUT_FS strain after 168h of culture in YPD medium with the glucose content of 5% and 0.26 g PET powder. Cultures were performed on a small scale in deep-well plates at 28 °C with continuous shaking at 200 RPM. $MnSO_4$- manganese (II) sulphate, $ZnSO_4$- zinc sulphate, $CuSO_4$- copper (II) sulphate, $CaCl_2$- calcium chloride, $MgSO_4$- magnesium sulphate. Stocks of the salts used in this study were prepared using hydrated salts, the molarity was converted concerning the dehydrated salt.

Statistical analysis was performed with the use of the Student's t-test. The amount of released degradation product in supplemented cultures was compared with the control samples (two-tailed; unequal variance). P-values labelled on the chart represent *- $p \leq 0.5$, **- $p \leq 0.01$, ***- $p \leq 0.005$. Errors bars are expressed as standard deviation from the mean.

Supplementary Table 1. Comparative table on PET degradation studies by cutinases and hydrolases.

Enzyme	Origin	Host	Specific name/mutation	Substrate	Degradation conditions	Degradation time	Amount of PET degradation products released	References
Cutinase	*Fusarium solani*	*Yarrowia lipolytica*	AJD 2 pAD CUT_FS	Semi-crystalline PET powder, 300 µm	Aerobic culture at 28 ºC, pH 8.5	192 hours	TPA: 1.51 gL^{-1} MHET: 0.45 gL^{-1}	This study
Hydrolase	*Fusarium oxysporum* LCH 1	-	-	PET yarn	Incubation with crude enzyme (activity 80 U) phosphate buffer at pH 7.0 in 30 ºC	168 hours	TPA: 16 μgml^{-1}	(Nimchua et al., 2007)
Hydrolase	*Fusarium solani*	-	-	PET yarn		168 hours	TPA: 9 μgml^{-1}	
Cutinase	Leaf-branch compost	*Clostridium thermocellum*	DSM1313::pHK-LCC	Amorphous PET film 0.25 µm thick	Anaerobic culture at 60 ºC, pH range 6.2-7.4	14 days	TPA+ MHET: 12 mM	(Yan et al., 2020)
Cutinase	*Saccharomonospora viridis* AHK190	*Escherichia coli*	Cut190_S226P/R228S (Cut190)*	BHET	Incubation with final enzyme concentration of 2 µM at 37 ºC, pH 7.5	2 hours	MHET: 275 µM	(Hantani et al., 2018)
Cutinase	*Thermobifida cellulosilytica*	*Escherichia coli*	ThC_Cut1	PET film	Incubation with 200 μgmL^{-1} at 50 ºC, pH 7.0	48 hours	TPA: 260 µM MHET: 20 µM	(Acero et al., 2013)
			ThC_Cut2				TPA: 85 µM MHET: 80 µM	
			Arg29Asn_Ala30Val				TPA: 400 µM MHET: 45 µM	
			Arg19Ser_Arg29Asn_Ala30Val				TPA: 375 µM MHET: 40 µM	
Cutinase	*Thermobifida fusca* KW3	*Escherichia coli*	TfCut2	PET fibres	Incubation with 50 µg of the purified enzyme at 65 ºC, pH 8.0	50 hours	TPA+MHET: 28 µM	(Wei et al., 2016)
			G62A				TPA: 72 µM MHET: 6.9 µM	
			G62A/I178V				TPA+MHET: 52 µM	

			G62A/I213S				TPA+MHET: 62 μM	
Cutinase	*Thermobifida cellulosilytica*	*Escherichia coli*	Thc_Cut1	PET membranes	Incubation with 5 μM of the purified enzyme at 50 ºC, pH 7.0	48 hours	TPA: 6 μM	(Gamerith et al., 2017)
			Thc_Cut2				TPA: 2.4 μM MHET: 4.2 μM	
			Thc_Cut1_PBM				TPA: 6.5 μM	
Cutinase	*Microbacterium oleivorans*	-	-	PET film	Incubation with final microorganism concentration 5 $mLcm^{-2}$ of PET at 35 ºC	15 hours	TPA: 20 nM MHET: 70 nM	(Yan et al., 2021)
	Thermobifida fusca				Incubation with final microorganism concentration 120 μgcm^{-2} of PET at 35 ºC		TPA: 27 nM MHET: 190 nM	
Cutinase	*Humicola insolens*	-	HiC	Post-consumer PET	Incubation with 1.0 $mg_{protein}mL^{-1}$ at 70 ºC at pH 7.0 with 220 gL^{-1} of PET	96 hours	TPA+MHET+BHET: 129 mM	(Eugenio et al., 2021)
Hydrolase	*Pseudomonas aestusnigri*	*Escherichia coli*	PE-H	Amorphous PET film	Incubation with 500 mM of the purified enzyme at 30 ºC at pH 7.4	48 hours	MHET: 4.1 mgL^{-1}	(Bollinger et al., 2020)
			PE-H Y250S				MHET: 5 mgL^{-1}	
			PE-H	Commercial PET bottle			MHET: 0.05 mgL^{-1}	
			PE-H Y250S				MHET: 0.12 mgL-1	
Hydrolase	HR29 bacterium	*Bacillus subtilis*	BhrPETase- CBS2ΔhrcA	Amorphous PET powder	Incubation with 5 μgmL^{-1} of the purified enzyme at 70 ºC at pH 8.0	20 hours	TPA: 2.47 mM MHET: 3.66 mM BHET: 0.17 mM	(Xi et al., 2021)
Cutinase	Leaf-branch compost		LCC				TPA: 2.4 mM MHET: 3.3 mM BHET: 0.15 mM	

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Production of PETase by engineered *Yarrowia lipolytica* for efficient poly (ethylene terephthalate) biodegradation

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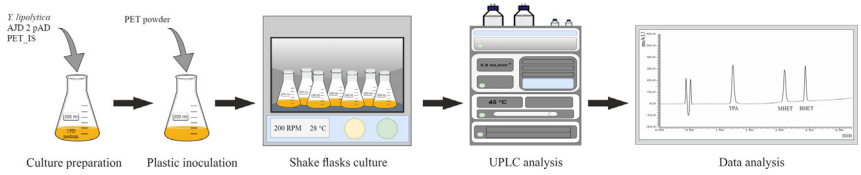
HIGHLIGHTS

- Overexpression of PETase in *Y. lipolytica* results in PET films degradation.
- Yeast strains used in this study are able to assimilate ethylene glycol.
- Significant surface defects of PET film were observed.
- Supplementation with 1 % olive oil improves PET hydrolysis.
- No PET pre-treatment required

GRAPHICAL ABSTRACT



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ABSTRACT

There has been a growing interest in poly(ethylene terephthalate) PET degradation studies in the last few years due to its widespread use and large-scale plastic waste accumulation in the environment. One of the most promising enzymatic methods in the context of PET degradation is the use of PETase from *Ideonella sakaiensis*, which has been reported to be an efficient enzyme for hydrolysing ester bonds in PET. In our study, we expressed a codon-optimized PETase gene in the yeast *Yarrowia lipolytica*. The obtained strain was tested for its ability to degrade PET directly in culture, and a screening of different supplements that might raise the level of PET hydrolysis was performed. We also carried out long-term cultures with PET film, the surface of which was examined by scanning electron microscopy. The efficiency of PET degradation was tested based on the concentration of degradation products released, and the results showed that supplementation of the culture with olive oil resulted in 66 % higher release of terephthalic acid into the medium compared to the mutant culture without supplementation. The results indicate the possibility of ethylene glycol uptake by both strains, and, additionally, the PETase produced by the newly engineered strain hydrolyses MHET. The structure of the PET film after culture with the modified strain, meanwhile, had numerous surface defects, cracks, and deformations.

1. Introduction

Plastics are now one of the most widely produced materials for commercial and industrial use. Those synthetic compounds are mainly used in the packaging, construction, automobile, and electrical industries. Globally, 367 million tonnes of primary plastics were produced in 2020, and poly(ethylene terephthalate) (PET) contributed to 8.4 % of this amount and was mainly used in the production of plastic bottles (Plastics Europe – Association of Plastics Manufacturers, 2020; PlasticsEurope Market Research Group (PEMRG)/Conversio Market &amp and Strategy GmbH, 2021). The high production level of plastics directly impacts the quantity of plastic waste generated nowadays. In 2020, 29 million tonnes of post-consumer plastic trash were collected in Europe, of which 34.6 % have been recycled. Encouragingly, the amount of plastic dumped in landfills fell from 12.9 to 6.9 million tonnes from 2006 to 2018, while the amount of plastic recycled doubled (PlasticsEurope Market Research Group (PEMRG)/Conversio Market &amp and Strategy GmbH, 2021).

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PET is a thermoplastic polymer categorized as non-biodegradable. Despite this, investigations into the feasibility of enzymatic degradation of PET have identified several enzymes capable of hydrolysing the ester bonds present in PET. The possibility of PET degradation has been confirmed by CALB lipase from *Candida antarctica*, cutinases from *Fusarium solani*, *Humicola insolens,* and *Thermobifida fusca*, and PETase from *Ideonella sakaiensis* (Carniel et al., 2017; Tanasupawat et al., 2016; Then et al., 2015; Tournier et al., 2020). The action of PET degrading enzymes is to break the internal bonds present in the polymer, resulting in the release of bis (2-hydroxyethyl) terephthalate (BHET), mono-(2-hydroxyethyl)terephthalic acid (MHET), terephthalic acid (TPA) and ethylene glycol (EG) (Mohanan et al., 2020). To improve the efficiency of PET degradation by enzymes, studies conducted so far have mostly focused on the use of genetic engineering methods to obtain higher enzyme secretion enabling further purification of the protein (Kim et al., 2020; Shi et al., 2021; Wang et al., 2020). Following reports that the most efficient enzymatic degradation of PET occurs at temperatures close to the glass transition temperature of 75 °C due to the greater mobility of the polymer chain, studies related to changing the structure of proteins to increase their thermostability have also been conducted (Ronkvist et al., 2009; Son et al., 2019). Considering the fact that the use of high temperatures for PET degradation is economically unfavourable, despite the ability of the produced proteins to act at high temperatures, it is necessary to look for further, superior solutions that are efficient and can be used on a large scale.

In this study, we overexpressed the PETase from *I. sakaiensis* in the yeast *Yarrowia lipolytica* and we optimized the PET degradation process by medium supplementation. In this study we performed PET degradation directly in the culture of the engineered strain of *Y. lipolytica*. Due to the capability for assimilation of atypical carbon sources which would allow for the assimilation of degradation products released in the process and good expression of the heterologous protein, the engineered yeast *Y. lipolytica* is a suitable organism for PET degradation.

2. Materials and methods

2.1. Microorganisms, media, and culture conditions

The yeast strains used in this study were AJD 2 pAD PET_IS (PETase overexpressing strain) and AJD2 (the control strain). LB medium was used for the growth of *Escherichia coli* (DH5α) strains. YPD (1 % yeast extract, 2 % peptone, 2 % glucose) medium was used for *Yarrowia lipolytica* inoculum preparation. For RNA isolation, a YNB (yeast nitrogen base) medium with 2 % glucose was used. The assimilation capacity of PET degradation products by *Y. lipolytica* was checked using a YPD medium containing 50 gL^{-1} glucose and the test compound. The final concentration of TPA and MHET in culture was 100 mgL^{-1}, while that of glycol was 10 gL^{-1}. Solutions of TPA and MHET were prepared by dissolving them in DMSO and methanol, respectively, and sterilized using 0.22 μM syringe filters (Merck Millipore). PET degradation experiments were performed in deep-well plates (DWP) and 0.3 L Erlenmeyer flasks. The growth curve analysis was conducted using a YPD medium with a dextrose content of 50 gL^{-1}. Semi-crystalline PET powder (particular size of 300 μm) and amorphous PET film (0.25 mm thickness) used as a plastic substrate in the media was purchased from GoodFellow Cambridge Limited (England) (ES306031/1; ES301445/7). PET material was previously sterilized by 10 min of UV irradiation. Supplements added to the medium to test their effect on degradation efficiency were sterilized using 0.22 μm syringe filters (Merck Millipore). Salt additives included: manganese (II) sulfate monohydrate ($MnSO_4$ x H_2O), zinc sulfate heptahydrate ($ZnSO_4$ x $7H_2O$), copper (II) sulfate pentahydrate ($CuSO_4$ x $5H_2O$), calcium chloride ($CaCl_2$), and magnesium sulfate heptahydrate ($MgSO_4$ x $7H_2O$). Salt stocks' molarity (50 mM) was calculated with consideration of their hydration level. Each supplement was added directly to the medium immediately before inoculation of the microorganisms and addition of plastic material. Tests were provided in 4 mL of YPD medium with glucose content of 50 gL^{-1} and the addition of 0.26 g of PET powder previously sterilized by 10 min of UV radiation. The addition of salt to the final concentration (1 mM and 2.5 mM) and olive oil (1 % and 2 %) was done before the inoculation of the culture. Control strain (AJD 2) and AJD 2 pAD PET_IS cultures were standardized to OD_{600} 0.1 and the experiment was conducted for 168 h (7 days); samples in triplicate were taken daily and the first sampling was after 72 h of culture. PET powder was added to the culture before the beginning of incubation. Based on the experiments performed in DWP we evaluated the most promising results and used them to scale up the process. For this purpose, we applied the conditions which gave a significant increase in the number of released degradation products on the larger laboratory scale. Experiments were performed in 0.3 L Erlenmeyer shake flasks in 30 mL of medium with addition of 2 g of PET powder previously sterilized for 10 min with UV light. Cultures were standardized to OD_{600} 0.5. Supplements and PET powder were added to the culture before incubation. Shake flask culture was performed at 28 °C at 200 RPM for 10 days (240 h) and the first samples were taken after 72 h of culture. All cultures conducted in this study were performed in three biological repetitions.

2.2. Strain construction

A codon-optimized PETase gene (GAP38373.1) from *I. sakaiensis* containing the XPR2 signal sequence from *Y. lipolytica* (915 bp) was cloned into the previously described pAD vector (Mirończuk et al., 2017) using SgsI and *Nhe*I FastDigest restriction enzymes and T4 DNA Ligase (Thermo Fisher Scientific). Digestion and ligation were conducted according to the manufacturer's protocols. *E. coli* transformation was executed using the standard protocol. Isolation of the obtained plasmid was done using the Plasmid Mini Kit (A&A Biotechnology) and the correctness of the overexpression cassette was verified by sequencing (Genomed, Poland). The constructed vector was linearized with the MssI FastDigest enzyme and the expression cassette was introduced to the *Y. lipolytica* genome based on rDNA integration using the lithium acetate-based transformation method described previously (Mirończuk et al., 2019), resulting in strain AJD 2 pAD PET_IS. Yeast genomic DNA isolation was performed with the Genomic Mini AX Yeast Spit Kit (A&A Biotechnology) and the verification of obtained clones was checked by standard PCR reaction.

2.3. Relative gene expression level and growth profile analysis

Y. lipolytica RNA isolation procedure and gene expression quantification were performed as described before (Kosiorowska et al., 2021). Primers used in qRT-PCR analysis to determine PETase expression level were qPET_IS_F (5′-GCCATGAAGCTCGCTACCGC-3′) and qPET_IS_R (5′-AGACGGCCATCAGACCACCC-3′) and the resultant product size was 109 bp. The relative expression level was normalized to the actin gene. Growth profiles were estimated using Spark Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). *Y. lipolytica* inoculum cultures grew for 24 h at 28 °C at 200 RPM. Culture cells were centrifuged and double rinsed in Milli-Q water. The experiment was conducted in 200 μL of YP medium with 50 gL^{-1} of glucose content. The effects of degradation products and solvents on yeast growth were evaluated on a YP medium with 50 gL^{-1} addition of glucose and relevant compounds: 100 mgL^{-1} TPA, 100 mgL^{-1}, MHET, 10 gL^{-1} EG, 10 gL^{-1} DMSO, 10 gL^{-1} MeOH. Experiments were carried out for 72 h with continuous orbital agitation at 28 °C. The initial OD was standardized to 0.05 and subsequent measurements of culture density were made at OD600 every 30 min of culture growth.

2.4. Analytical methods and statistical analysis

Samples obtained from cultures conducted in deep well plates (200 μL) and 0.3 L Erlenmeyer flasks (500 μL) supplemented with PET powder were centrifuged for 10 min at 10000 G and subsequently diluted in 99.9 % methanol (Chempur) followed by further centrifugation for 10 min at 10000 G at 10 °C. Terephthalic acid standard (TPA, Sigma-Aldrich) was prepared by dissolving in DMSO (Sigma-Aldrich). 2-Hydroxyethyl-terephthalic acid (MHET, AChemBlock) and bis(2-hydroxyethyl) terephthalate (BHET, Sigma) were

dissolved in 99.9 % methanol (P. P. H. STANLAB). Standards of compounds were prepared in stock with a final concentration of 10 gL^{-1} and were stored at −20 °C. Analysis of compounds released during PET degradation was performed by Ultra Performance Liquid Chromatography (UPLC) (Dionex-Thermo Fisher Scientific, UK) using a C18 type column (Hypersil Gold 100 × 2.1 mm, 3 μm) with a universal guard column (Hypersil Gold 10 × 2.1 mm, 3 μm). A gradient of two mobile phases was used during the analysis: acetonitrile with 0.1 % trifluoroacetic acid (*v*/v) and Milli-Q water with 0.1 % trifluoroacetic acid (v/v). The column temperature was 45 °C, the flow rate was 0.8 $mLmin^{-1}$ and the volume of the sample injected was 1 μL. Identification of analytes was performed with the UV detector at 243 ± 2 nm. The concentration of glucose and ethylene glycol was studied using a HyperREZ XP Carbohydrate H + 8 μm column (Thermo Scientific, Waltham, MA) and a refractive index (RI) detector (Shodex, Ogimachi, Japan). As the eluent 0.25 mM trifluoroacetic acid was used, the flow rate was 0.6 $mLmin^{-1}$ and the column temperature was 65 °C. The UPLC program used in this study was described in detail previously (Kosiorowska et al., 2022).

Statistical analysis was performed using Student's *t*-test to determine the significance of the influence of the supplements used in our study. The means of the control culture (without additives) were compared with the supplemented cultures (two-tailed, unequal variance). In addition, the results presented in the graphs include error bars that indicate the standard deviation of the samples calculated from the three biological replicates.

2.5. Scanning electron microscope analysis

Samples for examining plastic surfaces were derived from cultures conducted for 4 weeks at 28 °C at 200 RPM in 50 mL of YP medium containing 50 gL^{-1} of glucose, 2 g of PET film (GoodFellow ES301445/7, England), and from cultures performed in the same media with olive oil supplementation to a final concentration of 1 %. The analysis of the plastic surface was performed using a VEGA Tescan 3 scanning electron microscope and dedicated software. To prevent excessive accumulation of electric load plastic samples were coated with a thin layer of gold using a Cressington 108 Auto Sputter Coater with the following conditions: current 40 mA, sputtering time 60 s.

3. Results and discussion

3.1. Overexpression of I. sakaiensis PETase (PET_IS) and growth of the engineered Y. lipolytica strain

In recent years, the need to remove PET plastic wastes from the environment has been the subject of numerous studies. The enhancement of the natural plastic degradation processes can be improved by the use of genetic engineering methods. In our work, we used an unconventional yeast, *Y. lipolytica*, as the host organism; it possesses a GRAS (Generally Recognised as Safe) status, is able to assimilate atypical carbon sources such as alkanes and can grow in a wide pH range (Rzechonek et al., 2020). The codon optimized PETase gene (GAP38373.1) with the *Y. lipolytica* XPR2 signal sequence was expressed in the AJD 2 strain devoid of acidic (AXP) and alkaline (XPR2) extracellular proteases to eliminate the risk of heterologous protein degradation (Janek et al., 2020). The choice of this microorganism to secrete a PETase (6EQD_A) protein capable of poly (ethylene terephthalate) (PET) degradation (Yoshida et al., 2016) may carry the additional benefit of the ability of this type of yeast to assimilate atypical carbon sources. Over the past few years, this enzyme has been the target of numerous studies involving attempts to increase its heterologous expression in various host microorganisms (Kim et al., 2020; Shi et al., 2021) as well as modifying its structure to enhance protein stability (Chen et al., 2021; Han et al., 2017). PETase is an enzyme with an optimum temperature of 40 °C and exhibits activity at a wide pH range of 6–10 (Chen et al., 2021). It belongs to the class of hydrolases and its catalytic triad consists of the amino acids conventionally present in the active site of esterases: Asp-His-Ser (Fecker et al., 2018). The mechanism of hydrolysis of the ester bonds present in PET is based on the nucleophilic attack of serine on the carbonyl carbon present in this polymer (Feng et al., 2021). The products of PET hydrolysis are BHET (bis(2-hydroxyethyl) terephthalate), MHET (2-hydroxyethyl-terephthalic), TPA (terephthalic acid), and EG (ethylene glycol) (Mohanan et al., 2020).

First, to verify the functional expression of the *Y. lipolytica* strain expressing PETase from *I. sakaiensis*, the expression level of the cloned gene was analysed using the qRT-PCR method. Due to the use of a hybrid $UAS1B_{16}$-TEF promoter, whose highest activity was recorded after 24 h of growth (Blazeck et al., 2013), RNA isolation from the tested strain and from the control strain (AJD2) that was modified was performed on 24 h cultures. The relative expression level of the PET_IS gene is shown in Fig. 1A. This result indicates that the AJD 2 pAD PET_IS strain functionally expresses the target gene.

Considering the fact that genetic modifications of microorganisms can adversely affect their growth (Ko et al., 2020), we compared the growth profile of the AJD 2 pAD PET_IS strain with the control strain (AJD2). The experiment was conducted for 72 h in a YPD medium containing 50 gL^{-1} glucose, which was used at all further stages of this research. As shown in Fig. 1B there was no significant difference in the growth of the tested strain in comparison with the control strain. Both strains presented an extended lag phase occurring up to 15 h of culture and an exponential phase persisted up to 40 h of culture. In agreement with previous studies, no significant delay in the growth of the modified strains was observed (Dobrowolski and Mirończuk, 2020; Kosiorowska et al., 2021).

3.2. Capability of the engineered Y. lipolytica strain to assimilate PET degradation products

Consistent with reports on the capability of *Y. lipolytica* yeast to assimilate atypical carbon sources (Mirończuk et al., 2018), we tested the capability of the yeast used to grow on PET degradation products. These studies were crucial to verify that the possible uptake of hydrolysis products would not disturb the actual concentration of degradation products measured in the culture supernatant. Experiments were carried out in YPD medium supplemented with 50 gL^{-1} glucose supplemented with TPA, MHET, EG, DMSO or MeOH as required. Regarding the fact that BHET is released during PET degradation in negligible amounts (Vertommen et al., 2005), and was not detected in our preliminary studies, it was not included in the tests. The results shown in Fig. 2A demonstrate that neither the control strain nor the modified strain takes up TPA. A slight increase in the concentration of this compound in the medium was observed; however, considering the duration of the culture (168 h) this increase is associated with the evaporation process. This increase is not statistically significant. The glucose was consumed up until 48 h of culture and its uptake was identical for both strains. These results are in contrast with previous studies performed by Costa et al. in 2020, where TPA consumption by *Y. lipolytica* IMUFRJ 50682 in YPD media was observed. However, the authors used a YPD medium with lower glucose content. In addition, they observed a decrease in TPA assimilation in the glucose-supplemented medium compared to the medium without the additional carbon source (da Costa et al., 2020).

To investigate the ability of MHET hydrolysis by AJD 2 and AJD 2 pAD PET_IS strains, an experiment using media supplemented by this compound was carried out. As shown in Fig. 2B, the AJD 2 pAD PET_IS strain completely hydrolysed MHET to TPA after only 96 h of culture. In the control culture, no decrease in the MHET concentration was observed and the very low concentration of TPA detected is due to the spontaneous hydrolysis of MHET. The results indicate that a PETase from *I. sakaiensis* produced by the modified *Y. lipolytica* strain is capable of efficient hydrolysis of MHET without the support of the other overexpressed hydrolases. This finding concurs with other studies which have shown that the PETase enzyme requires additional MHETase enzyme responsible for MHET hydrolysis during the PET degradation process (Joo et al., 2018; Yoshida et al., 2016).

It can be seen from Fig. 2C that both the AJD 2 and AJD 2 pAD PET_IS strains have the capability to assimilate ethylene glycol (EG) under the

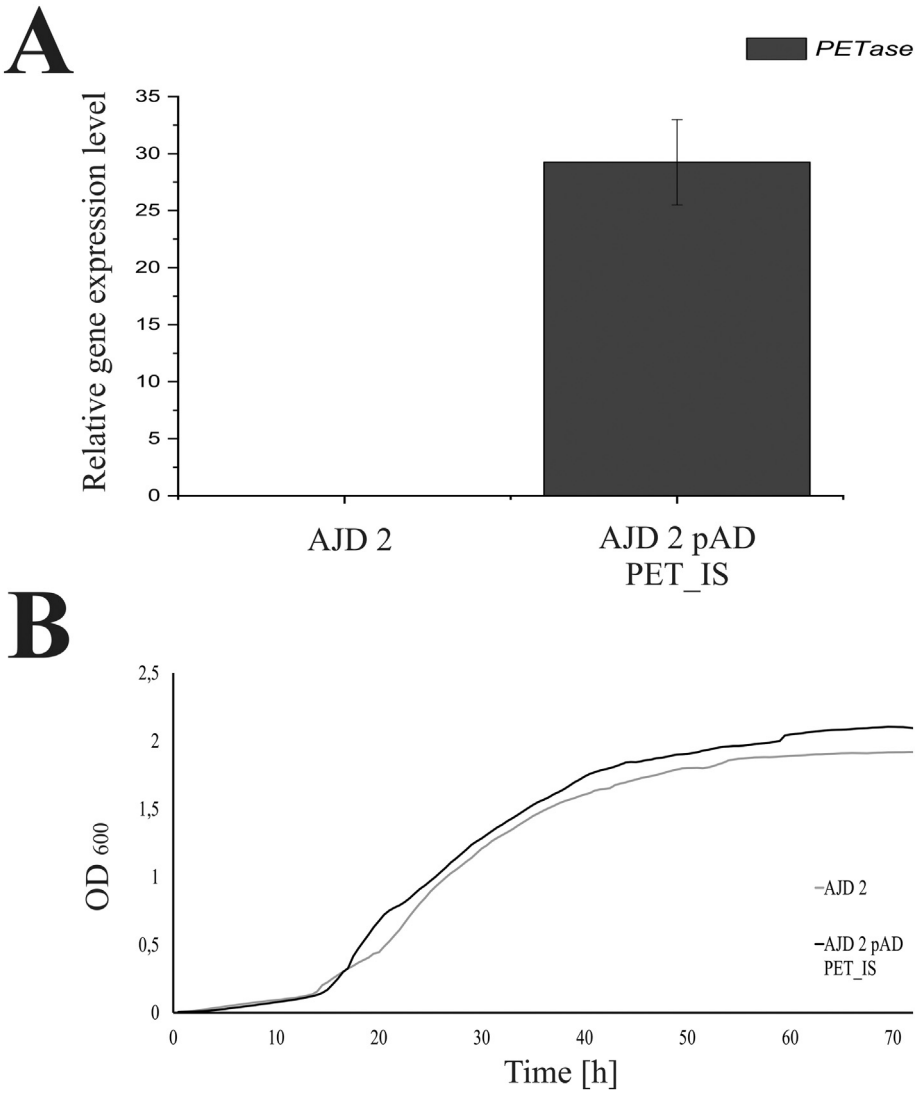

Fig. 1. Relative PETase gene expression level (A) in the obtained *Y. lipolytica* AJD 2 pAD PET_IS strain and control strain (AJD 2). Growth curve analysis (B) between AJD 2 and newly derived strain in YPD medium containing 50 gL^{-1} of glucose.

given conditions. It was observed that EG is taken up concomitantly with glucose, which is consumed with lower rates in contrast to the medium supplemented with TPA or MHET. To date, there are no studies regarding the capacity of yeast to assimilate EG, and metabolic pathways for natively existing pathways have been found only in bacterial species (Mückschel et al., 2012; Pandit et al., 2021).

Due to the use of compounds that have not been previously tested for their effect on the growth of *Y. lipolytica* yeast and the fact that TPA and MHET components were dissolved in DMSO and MHET respectively, whose toxicity toward microorganisms has been confirmed (Sadowska-Bartosz et al., 2013; Vartiainen et al., 2019), growth curves were constructed using the medium supplemented with an appropriate concentration of each of the tested supplements. As can be seen from S. Fig. 1, the applied concentrations of compounds and solvents do not have a negative effect on the growth of either the control or the tested strain.

3.3. Process optimization by the engineered Y. lipolytica strain

A key limitation of PET degradation by PETase is the low secretion level of this hydrolase by the *I. sakaiensis* bacterium, which directly affects the efficiency of the process. The literature concerning the use of genetic engineering methods demonstrates a variety of approaches in the host-organism selection that may alter the secretion level of this protein. To date, successful expression of PETase protein from *I. sakaiensis* has been performed in green algae (Kim et al., 2020), *Escherichia coli* (Shi et al., 2021), and *Bacillus subtilis* (Xi et al., 2021). The purpose of our study was to use *Y. lipolytica* as a host organism for extracellular PETase production and also to test the effect of supplementation on the PET degradation rate in the yeast culture. To determine the modified *Y. lipolytica* strain's ability to degrade PET, we first provided a small-scale experiment in deep-well plates (DWP). Small scale experiments were also used for estimation of the influence of supplementation of various salt and olive oil concentrations on degradation efficiency.

Following the exclusion of TPA assimilation in YPD medium with 50 gL^{-1} glucose, we performed degradation studies on PET powder added to cultures with the modified *Y. lipolytica* strain. In parallel, we investigated the effect of supplements such as inorganic salts and olive oil at different concentrations on the PET degradation level measured in the form of released degradation products. In agreement with a previous experiment, where we observed hydrolysis of MHET to TPA by the enzyme PETase and assimilation of ethylene glycol with the applied substrate composition, MHET and EG were not detected, and we determined the effect of supplementation from the measured concentration of TPA in the supernatants. The BHET compound was also not observed in any of the tested samples. The results are shown in Fig. 3.

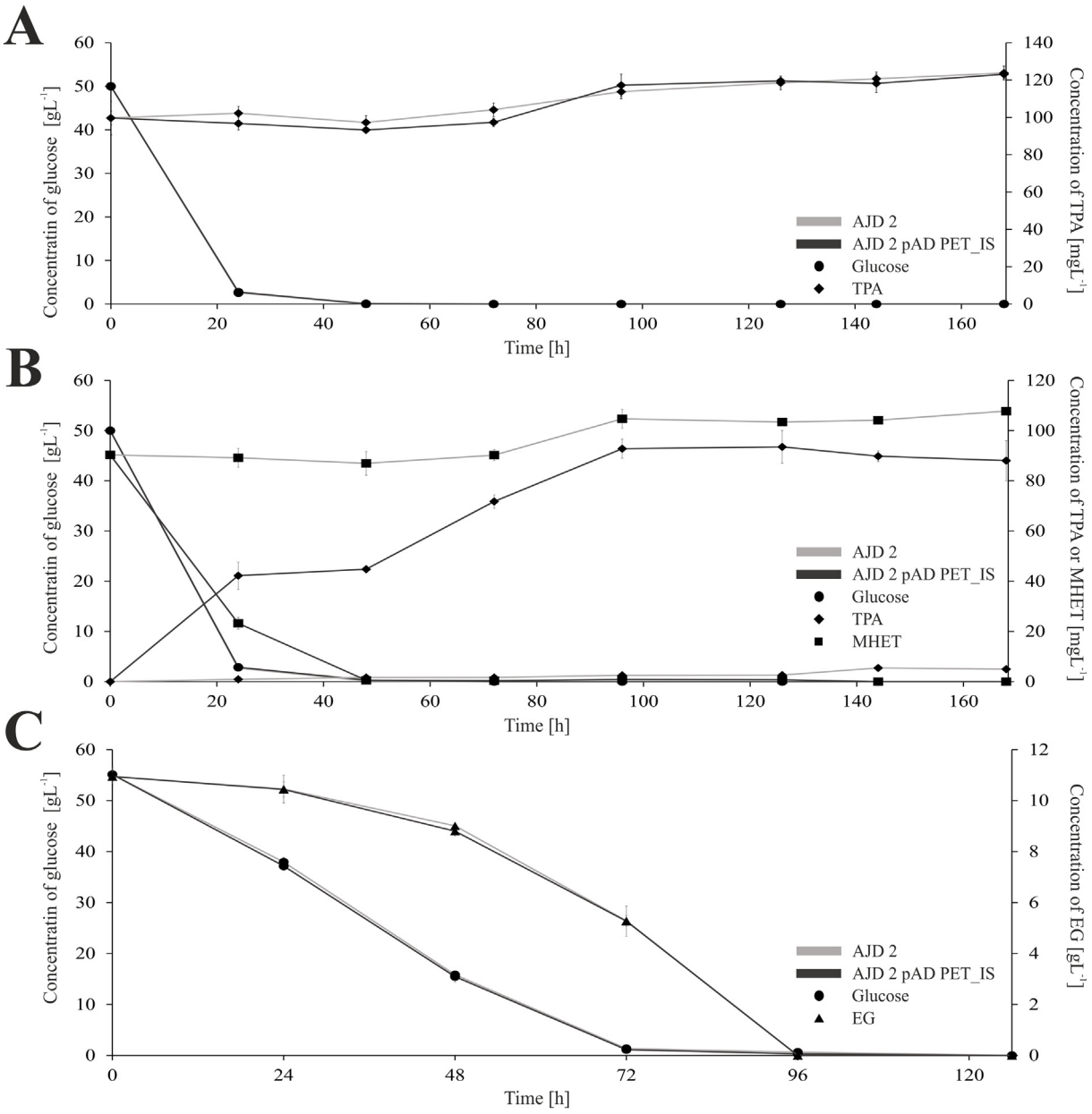

Fig. 2. Growth analysis of AJD 2 control strain and AJD 2 pAD PET_IS mutant with respect to PET hydrolysis products: panel A presents results derived from culture in the medium containing TPA, panel B contains results from cultures with the addition of MHET compound, panel C shows data from the culture with EG additive. Each graph also presents the profile of glucose depletion in the medium.

The supplements used in this study were selected based on previous scientific reports regarding the positive effects of salt ions on esterase enzymatic activity (Liu et al., 2019). Studies conducted on esterases also indicate that salt ions such as Mg^{2+}, Zn^{2+}, and Mn^{2+} used in concentrations of 2.5 mM and 5 mM enhance their enzymatic activity (Kawai et al., 2014; Metin et al., 2006; Park et al., 2021). Scientific literature also points to the increase in the stability of hydrolytic enzymes by Ca^{2+} and Mg^{2+} ions (Gricajeva et al., 2021; Urbanek et al., 2021). In our research, the use of *Y. lipolytica* as a host organism offers additional opportunities for supplementation in terms of induction of native lipase production using olive oil (Papanikolaou et al., 2007), for which the capacity for PET hydrolysis was previously described (Carniel et al., 2017). Moreover, lipases can increase the hydrophilicity of synthetic polyesters, thus facilitating access of the PETase enzyme to the hydrophobic substrate (Kim and Song, 2010). A further advantage of this yeast is its ability to assimilate atypical carbon sources, which allows the assimilation of one of the degradation products released into the medium (Fig. 2C).

In the first phase of our study on PET degradation by the AJD 2 pAD PET_IS strain, we focused on the screening of the supplements that could enhance the hydrolysis of this polymer during cultivation. First, we performed small-scale experiments for 168 h, in which we measured the concentration of TPA released to the supernatant since 72 h of cultivation. The daily increase of terephthalic acid in the collected samples is shown in S. Fig. 2. The first measurable concentrations of TPA in the supernatants occur after 120 h of culture, which is associated with an increase in the pH of the culture to the pH optimal for PETase activity (S. Fig. 4A). In the experiments performed on the AJD2 strain, which was tested for PET hydrolysis under identical conditions as the AJD2 pAD PET_IS strain, the presence of TPA in the supernatants was not recorded in any case. Additionally, we did not note the BHET compound in any of the tested samples. This result is contrary to reports (da Costa et al., 2020) where the tested *Y. lipolytica* strain hydrolysed PET, resulting in the release of approximately 23 mgL^{-1} MHET after 96 h of culture in YPD with amorphous PET.

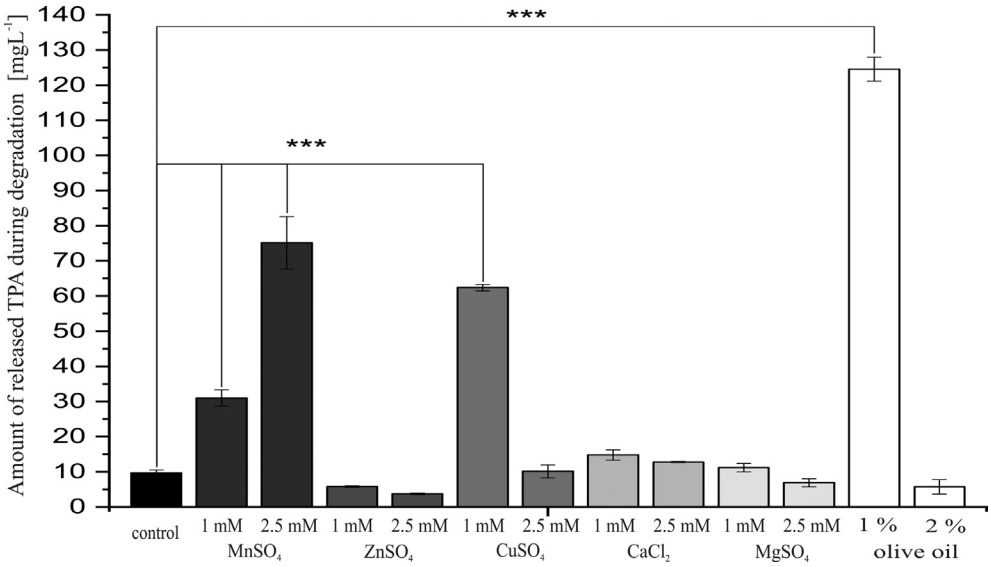

Fig. 3. Screening of the influence of the various supplementary additives on the PET degradation level in the cultures carried out in deep well plates for 168 h. Statistical analysis was performed using Student's *t*-test; error bars represent standard deviation. All experiments were performed in triplicate.

The results shown in Fig. 3 present the measured TPA concentration in the AJD 2 pAD PET_IS culture. The control labelled in the graph refers to the amount of terephthalic acid released to the media during cultivation with the engineered *Y. lipolytica* (AJD 2 pAD PET_IS) strain without supplementation and was 10 mgL^{-1}. The statistical analysis of the results was performed using Student's *t*-test, where the results from the supplemented cultures were compared to the control culture. PET degradation was significantly enhanced by the addition of $MnSO_4$ salt at both concentrations used (1 mM and 2.5 mM), where the measured amount of TPA was 30 mgL^{-1} and 75 mgL^{-1}, respectively. The hydrolysis of PET was also significantly influenced by the addition of 1 mM $CuSO_4$, where the concentration of TPA was 62 mgL^{-1}. In the experiment conducted with a higher concentration of this salt (2.5 mM), no positive effect on degradation efficiency was observed. The other additions of salts to the culture medium did not significantly affect the level of PET decomposition. Moreover, the addition of $ZnSO_4$ reduced it in both concentrations used. The data obtained are not surprising in the context of previous scientific reports on the effect of salt on esterase activity. The established effect of culture supplementation with $MnSO_4$ salt concurs with the experiments performed by Senga et al. in 2020. The relative activity against PBSA by examined esterase showed that in the presence of 2.5 mM Mn^{2+} ions it reached 65 % of the activity measured in 2.5 mM Ca^{2+} (Senga et al., 2020). A statistically insignificant increase in TPA concentration with the addition of $CaCl_2$ and $MgSO_4$ was observed. The highest observed amount of TPA released occurred with the addition of olive oil to a final concentration of 1 % in the culture and was 124 mgL^{-1}. This could be related to the induction of lipase production, which could act synergistically with secreted PETase to increase the level of PET degradation. In a previous study, it was noted that the cooperative action of cutinase and lipase effectively degraded PET (Liu et al., 2018). Interestingly, the addition of this supplement at a final concentration of 2 % had

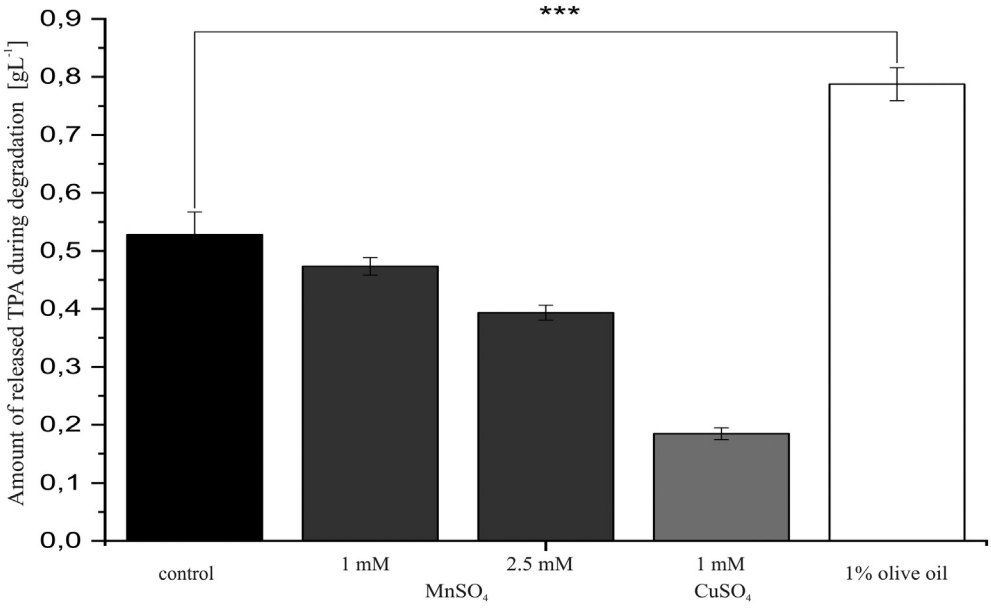

Fig. 4. Amount of TPA released during PET degradation process carried out in 0.3 L Erlenmeyer shake cultures with or without supplementation. Cultivation was carried out for 240 h in YPD medium containing 50 gL^{-1} of glucose and 2 g of PET powder at 28 °C at 200 RPM. Student's t-test was used for the statistical analysis; error bars represent standard deviation. All cultures were performed in triplicate.

no effect on the degradation efficiency. Supplementation of the culture with olive oil to a final concentration of 1 % resulted in a statistically significant increase in the amount of TPA released. Therefore, the results obtained may also indicate the influence of biosurfactants produced by *Y. lipolytica*, where at a lower olive oil concentration their production of them is induced, and at a higher concentration the phenomenon of their affiliation with the cell wall occurs (Fontes et al., 2010). The production of biosurfactants is closely related to the assimilation of hydrophobic products. The study by Fontes et al., 2010 also confirmed that high glucose concentration in the medium promotes the production of natural surfactants. Other researchers have used carbohydrate media supplemented by vegetable oils for surfactant production in the *Candida lipolytica* yeast strain (Sarubbo et al., 2007). Previous research has shown that surfactant addition improves PET degradation (Samak et al., 2020).

Subsequent to screening for potential supplements that could enhance PET degradation efficiency in the yeast culture, we made an attempt to scale up the degradation process. For this purpose, we used 0.3 L Erlenmeyer flasks and culture conditions where a statistically significant effect on PET hydrolysis was observed. The culture was conducted for 240 h and the results of TPA concentration at the final checkpoint are shown in Fig. 4. The samples were collected each day starting at 72 h and the daily increase in released product amount is shown in S. Fig. 3. Considering the pH as an important factor affecting the enzyme activity, it was monitored for each checkpoint. From S. Fig. 4B, it can be seen that the pH was stable at 8 throughout the sampling period for both AJD 2 and AJD 2 pAD PET_IS strain. Consistent with the cultivation carried out in deep well plates, we did not observe PET degradation by the AJD 2 strain, and TPA was observed as the sole hydrolysis product in the strain AJD2 pAD-PET_IS. The supplements used were selected based on a previous screening experiment where a statistically significant effect on PET degradation was observed in cultures containing 1 mM and 2.5 mM $MnSO_4$, 1 mM $CuSO_4$, and 1 % olive oil. The control culture labelled in the graph refers to AJD 2 pAD PET_IS culture in a medium without supplementation. The obtained results of salt supplementation in flask cultures did not confirm their positive effect on the degradation process. In the 2 pAD PET_IS culture without supplementation, the measured amount of released TPA was 0.53 gL^{-1}, while the highest amount of TPA released in the case of salt supplementation was obtained in the medium containing 1 mM $MnSO_4$ and was 0.47 gL^{-1}. When cultures containing 2.5 mM $MnSO_4$ and 1 mM $CuSO_4$ were grown, the TPA concentration was 0.39 gL^{-1} and 0.18 gL^{-1}, respectively, which is 1.35- and 2.9-fold lower than in the control culture. Contrary to the lack of a positive effect of salt on PET degradation when scaling up the process, the addition of olive oil to a final concentration of 1 % in the culture did have a statistically significant effect on the degradation level of this polymer. The concentration of TPA was 0.88 gL^{-1}, which is 66 % higher than in the culture without olive oil added. The non-positive effect of the addition of salts to the growth medium for cultures conducted in 0.3-L Erlenmeyer flasks may be related to the aeration conditions and cell growth rate. In comparison to the small scale, the yeast cells had a better environment for growth and the salts under these conditions had the opposite effect to the previously observed results. Particularly important for the growth of *Y. lipolytica* is aeration, the crucial effect of which has been described before (Mirończuk et al., 2019). Analysis of the daily increment of a degradation product is shown in S. Fig. 3. Cultures without supplementation to salt-supplemented cultures show no remarkable differences in the rate of growth of the amount of the TPA. The rise of the compound concentration is gradual, and the highest daily increase is observed on the last day of culture. Regarding the olive oil-supplemented culture, fundamentally different dynamics of TPA accumulation in the supernatant can be observed. In this case, three points of spiking increase of terephthalic acid concentration can be noted: between 96 h and 120 h, 120 h and 144 h, and on the last day of culture.

The degradation of PET plastic material during the cultivation of a microorganism capable of secreting PETase enzyme has not been the subject of much research. Studies published so far have been mostly focused on the production, isolation, and purification of PETase for further incubation with the plastic under optimized conditions. In this research, the novel degradation of PET in *Y. lipolytica* yeast culture can provide an interesting alternative in studies on the hydrolysis of this polymer. In the method proposed, purification of the enzyme is not required, the steady pH of the culture corresponds with the optimum for the enzyme action, and the low temperature of cultivation ensures the stability of the protein. Moreover, as illustrated previously, *Y. lipolytica* can assimilate ethylene glycol as a carbon source, enabling yeast to grow despite the depletion of the main carbohydrate source. In previous studies on PET degradation by PETase from *I. sakaiensis*, the number of degradation products released varied depending on the applied incubation conditions and the concentration of enzyme employed. In the first study reporting the promising capabilities of PETase, approximately 0.09 mM TPA and 0.2 mM MHET were obtained (Yoshida et al., 2016). These results were noted after 18 h of incubation at 30 °C and a pH of 7.0. An interesting study was conducted by Chen et al. in 2021, where PETase was expressed in *E. coli*. Incubation on PET film with purified enzyme caused the release of approximately 0.12 mM of total TPA and MHET after 6 days of incubation at 30 °C with PET. Interestingly, when a temperature of 40 °C was used, the total amount of released degradation products was slightly higher and was about 0.13 mM (Chen et al., 2021). Conversely, in the study of Shi et al., 2021, PETase containing the signal peptide pelB after 48 h of incubation with PET powder resulted in the release of a total of 531 μM of TPA and MHET. From the measured amount, approximately 400 μM was TPA and 130 μM was MHET, which is 64 mgL^{-1} and 30 mgL^{-1}, respectively. Intriguingly, no significant increase in the amount of degradation products was observed between the established control points in this study (18 h, 24 h, and 48 h) (Shi et al., 2021). A further study with PETase from *I. sakaiensis* performed by Son et al. in 2019 showed that during incubation of the enzyme with PET at 30 °C after 72 h, 11.5 μM of total TPA and MHET was released, whereas, at 40 °C, the total amount of degradation products was 8.7 μM. Enzyme stability during PET incubation for 10 days at both temperatures used demonstrated that PETase maintains its activity throughout the experiment (Son et al., 2019). A different study on PET degradation showed that, using 5 μg of purified PETase enzyme, approximately 2.5 mM TPA (400 mgL^{-1}) after 48 h of PET film incubation at 30 °C was released (Ma et al., 2018). Among the studies devoted to PET degradation by PETase, Kim et al., 2020 reported a very interesting application, whereby green microalgae were used as a host organism for production of this hydrolase. 30 mg of PET powder was incubated in cell lysate for 4 weeks at 30 °C, resulting in a quantity of TPA of 9.12 mg (Kim et al., 2020).

Notably, in addition to PETase, another promising enzyme for PET biocatalytic recycling research is cutinase produced by such microorganisms as: *Fusarium solani*, *Fusarium oxysporum*, *Thermobifida fusca*, *Saccharomonospora viridis* (Furukawa et al., 2019; Groß et al., 2017; Nimchua et al., 2007). Similar to PETase investigations described above, studies conducted using different cutinases relied on employing purified protein and executing the reaction under the most optimal conditions for enzyme action. Interesting results were obtained in a study comparing the action of two cutinases from *Fusarium solani* and *Fusarium oxysporum*, where enzymes with activity of 80 U and PET yarn as substrate, were used. Regarding the first enzyme, after 168 h of reaction at pH 7.0 and temperature 30 °C, approximately 9 $ugmL^{-1}$ TPA was released, while hydrolysis of PET by *F. oxysporum* resulted in the release of approximately 16 $ugmL^{-1}$ TPA. Intriguing findings were reported by Eugenio et al. in 2021 during a study on cutinase from *Humicola insolens*, where post-consumer PET was used as a substrate. PET flakes have been incubated for 96 h with 1.0 $mg_{protein}mL^{-1}$ at 70 °C in pH 7.0, and the total amount of hydrolysis products released (TPA, MHET and BHET) was 129 mM (Eugenio et al., 2021). Another noteworthy study is the leaf-branch compost cutinase, described by Sulaiman et al., 2014, which inherently exhibits high thermostability and the ability to hydrolyze PET, and within 24 h caused a loss of about 5 mg of PET film weight (initial PET weight 20–25 mg) at 70 °C (Sulaiman et al., 2014). However, other researchers, in their work on LCC, proved that the protein is susceptible to aggregation in its native state, but this effect can be

inhibited by protein glycosylation (Shirke et al., 2018) . In this work, the LCC cutinase was expressed in *Pichia pastoris* (*Komagataella phaffii*), and the results of PET film degradation indicated that after 48 h the polymer weight loss was more than 95 %. The synergistic effect of hydrolytic enzymes produced by *Microbacterium oleivorans* and cutinase from *Thermobifida fusca* was recently observed by Yan et al., 2021. Here, it was proven that the combination of *M. oleivorans* culture and 120 μgmg^{-1} of cutinase from *T. fusca* can contribute to more efficient degradation of PET films as low as 35 °C, with 47 nM TPA and 330 nM MHET released during 15 h of incubation (Yan et al., 2021).

In another study, various hydrophobic or hydrophilic monomers (TBMA, HEMA, DMAEMA, MA) were used to improve the catalytic

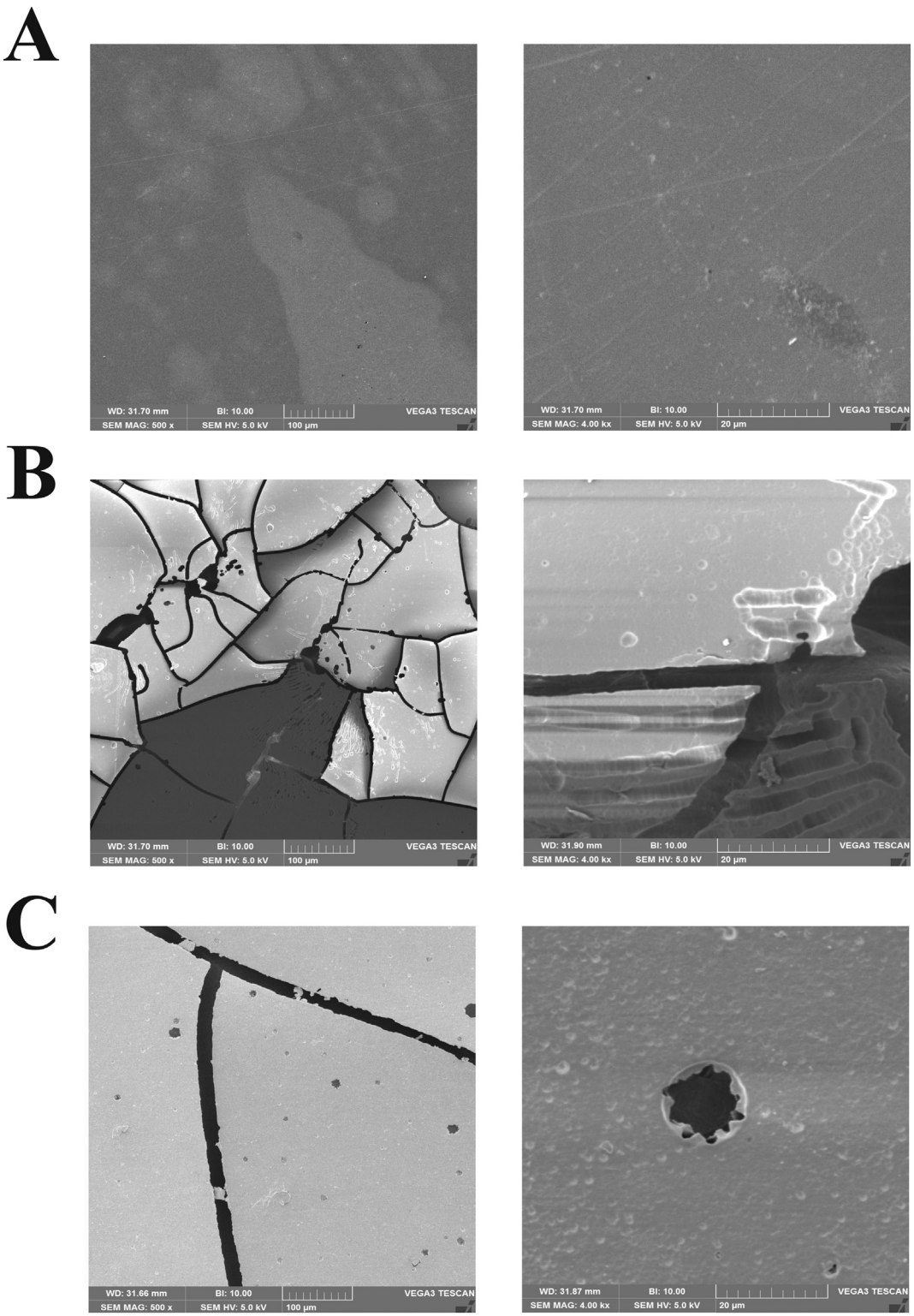

Fig. 5. Images of PET film surface derived from scanning electron microscope after 4 weeks of PET film incubation of control strain (AJD 2) (A) and AJD 2 pAD PET_IS (B) in YP medium containing 50 gL^{-1} of glucose. Fig. C presents PET film obtained in the mutant culture in a medium supplemented with olive oil. All cultures were incubated at 28 °C at 200 RPM. Detailed description (magnification, scale) is shown under every presented sample.

performance of PETase and the protein thus conjugated was tested with PET film at 40 °C. The best PET hydrolysis results were obtained using TBMA-PETase and DMAEMA-PETase, where the total amount of TPA and MHET released was 82.5 μM and 77.5 μM, respectively. Artificial intelligence was also used in the development of the GRAPE (*greedy accumulated strategy for protein engineering*) algorithm, with the help of which a DuraPETase mutant was obtained, possessing as many as 10 mutations in the amino acid chain. The generated PETase mutant, after 10 days of incubation with PET film, resulted in the release of 3.1 mM total PET degradation products (TPA, MHET, BHET) (Cui et al., 2021). In the work of Liu et al., 2022, this variant (DuraPETase) underwent further modifications in the amino acid chain to increase the thermostability of the enzyme (N233C/S282C/H214S/S245R). When degrading the amorphous PET powder at 60 °C, the total amount of degradation products released after 96 h with the purified DuraPETase-4 M enzyme was 15.8 mM, which was 2.77 fold higher than the initial mutant tested under the same conditions (Liu et al., 2022).

Protein engineering has also targeted cutinases, and among the most promising cutinase modifications, was the one made by Tournier et al., 2020. In this work, the LCC cutinase was subjected to a series of mutations, two of which, named WCCG (F243I/D238C/S283C/Y127G) and ICCG (F243W/D238C/S283C/Y127G), caused depolymerization of more than 90 % of the PET substrate in 10.5 h and 9.3 h at 72 °C, respectively (Tournier et al., 2020). The comparison of PET degradability by enzymes described above, with respect to reaction conditions, degradation time and amount of hydrolysis products released can be found in Supplementary Table 1.

3.4. PET film surface morphology analysis

After cultivation of *Y. lipolytica* strains in medium with the addition of PET powder, we used a more difficult to degrade type of substrate, i.e. PET film. For that purpose, the medium supplemented with olive oil to a final concentration of 1 % in the medium was used. To compare the effect of supplementation, a culture without supplements was conducted in parallel. The addition of PET film to the medium was 2 g of equally divided film fragments with dimensions of 1 cm × 1 cm. The collected film samples were analysed using a scanning electron microscope (SEM). Images showing the performance of the AJD 2 pAD PET_IS strain are shown in Fig. 5. The visual changes in the appearance of the films were also noticeable in the samples without SEM analysis and are shown in S. Fig. 5. No significant damage to the plastic surface was observed in the culture of strain AJD 2 at either of the magnifications used, 500 x or 4000 x (Fig. 5A). The surface of the film after culture with the AJD 2 pAD PET_IS strain in a medium with no olive oil added is shown in Fig. 5B. Both magnifications used demonstrate extensive damage to the surface of the material. The film is significantly cracked and numerous gouges can be seen on the surface. The depicted fragments also exhibit cavities in the plastic material. Fig. 5C presents the PET film after culture with a modified strain of *Y. lipolytica* in the medium supplemented with olive oil. The structure of the film obtained from this culture exhibits visible craters of irregular shape in parallel with the cracks. It can also be noted that the frequency of the breaks on this PET material is lower than in the films obtained from the experiment using the same strain but without supplementation (Fig. 5B). The films collected after long-term culture with yeast strains were also subjected to an estimated mass loss analysis (Supplementary Fig. 6). According to the data obtained, the addition of olive oil to the culture medium caused not only more corrosion on the film surface but also a greater polymer mass loss of 53.05 % of the initial mass. In the case of the substrate without supplementation, there was a 44.74 % decrease in film weight. For the culture of the control strain (AJD 2), the mass loss was imperceptible.

The analysis of changes in the polymer surface after PETase treatment plays a crucial role in determining the PET degradation level. The results obtained in our study correspond with previously reported data on plastic surface corrosion arising using the PETase enzyme (Shi et al., 2021; Yoshida et al., 2016). However, due to the extended culture of the *Y. lipolytica* modified with PET film, degradation level in our study is more apparent. Interestingly, in the 6-day incubation of PETase enzyme at 40 °C at pH 9.0 with PET film in the study by Chen et al. in 2021, changes in the plastic structure were barely noticeable (Chen et al., 2021). Considering the culture time, similar to our research, Kim et al. in 2020 incubated PET film for 4 weeks in the cell lysate of PET-producing green microalgae. In their study, the surface changes are slightly different due to the absence of visible cracks in the material, but numerous depressions and cavities could also be seen (Kim et al., 2020).

In summary, here we present the possibility to use an unconventional yeast strain as a host organism for the extracellular production of PETase. The methodology used in this work allowed us to determine the ability of the obtained strain to degrade PET, and its universal applicability in research on the degradation of this polymer allows us to compare the results with other reports on the discussed topic.

4. Conclusion

The present study showed that the modified *Y. lipolytica* yeast is capable of PET degradation directly in the culture. The obtained data showed that olive oil addition significantly improves the PET hydrolysis efficiency. However, we also observed that the addition of salt can reduce the degradation rate of the polymer, and the optimisation of the substrate composition has a key role in the degradation of PET by the method used. Furthermore, our study showed that *Y. lipolytica* cannot assimilate TPA; nevertheless, with the medium composition used, it can take up ethylene glycol. The applied novel approach has a great potential for further PET degradation during yeast culture growth.

CRediT authorship contribution statement

Katarzyna E. Kosiorowska: Investigation, Validation, Formal analysis, Visualization, Writing - original draft. **Antonio D. Moreno:** Supervision, Writing - review & editing. **Raquel Iglesias:** Supervision, Writing - review & editing. **Karol Leluk:** Investigation, Visualization. **Aleksandra M. Mirończuk:** Conceptualization, Supervision, Writing - review & editing, Project administration, Funding acquisition.

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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.

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Appendix A. Supplementary data

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Supplementary material

Production of PETase by engineered *Yarrowia lipolytica* for efficient poly(ethylene terephthalate) biodegradation

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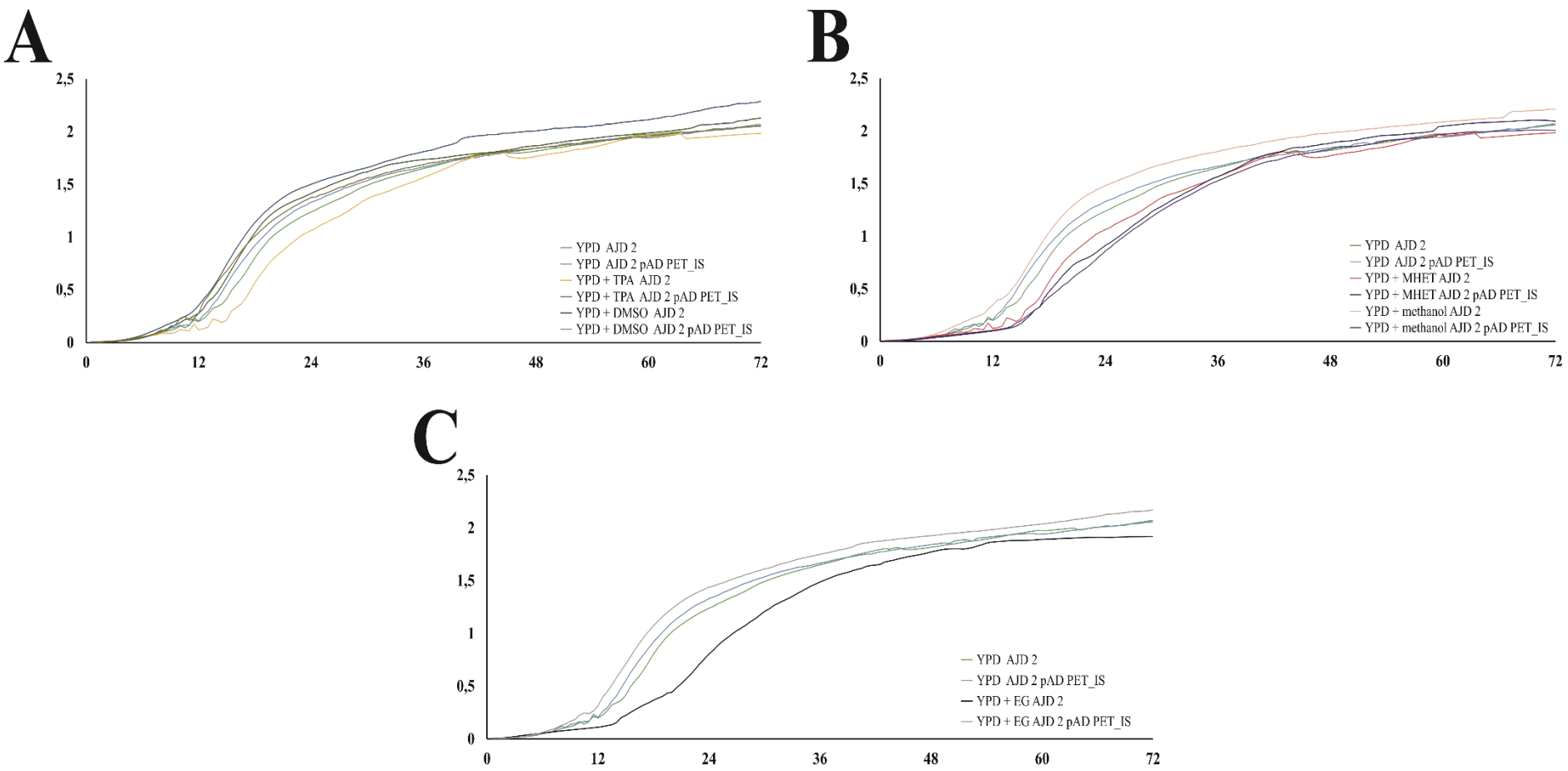
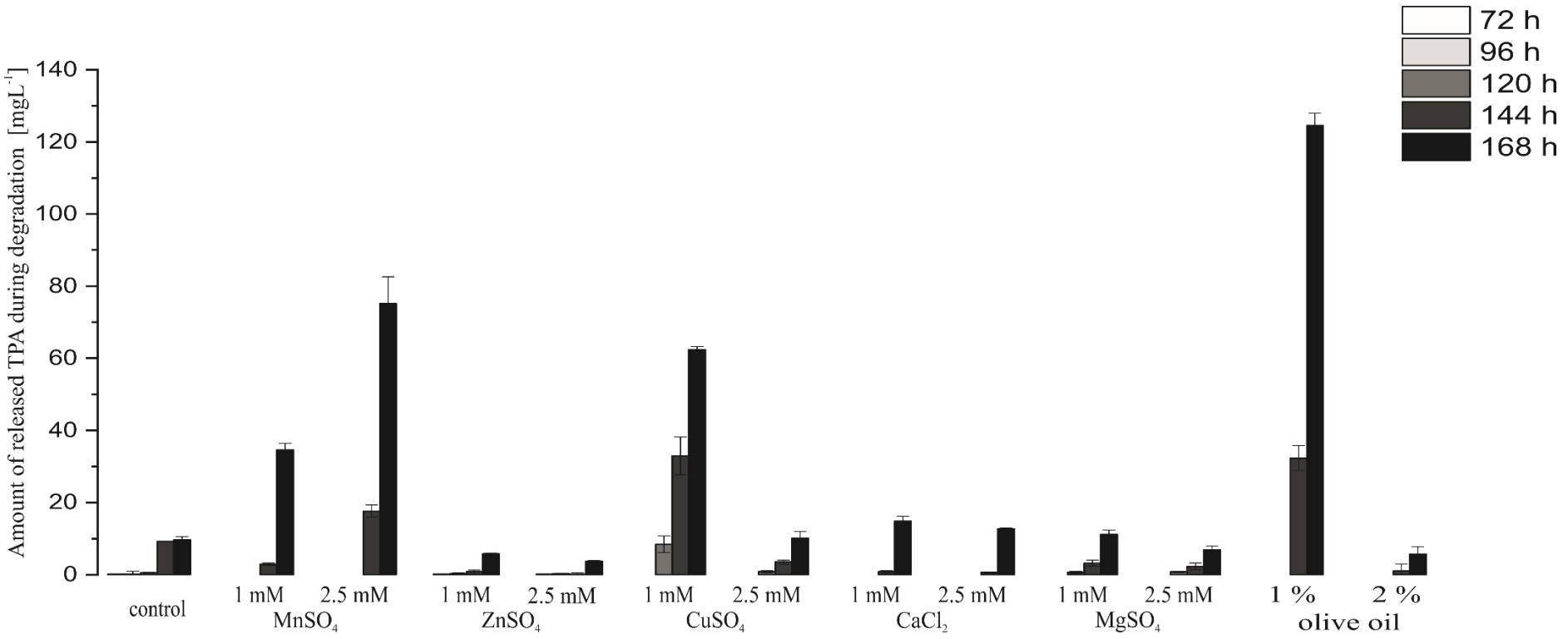


Supplementary Fig. 1

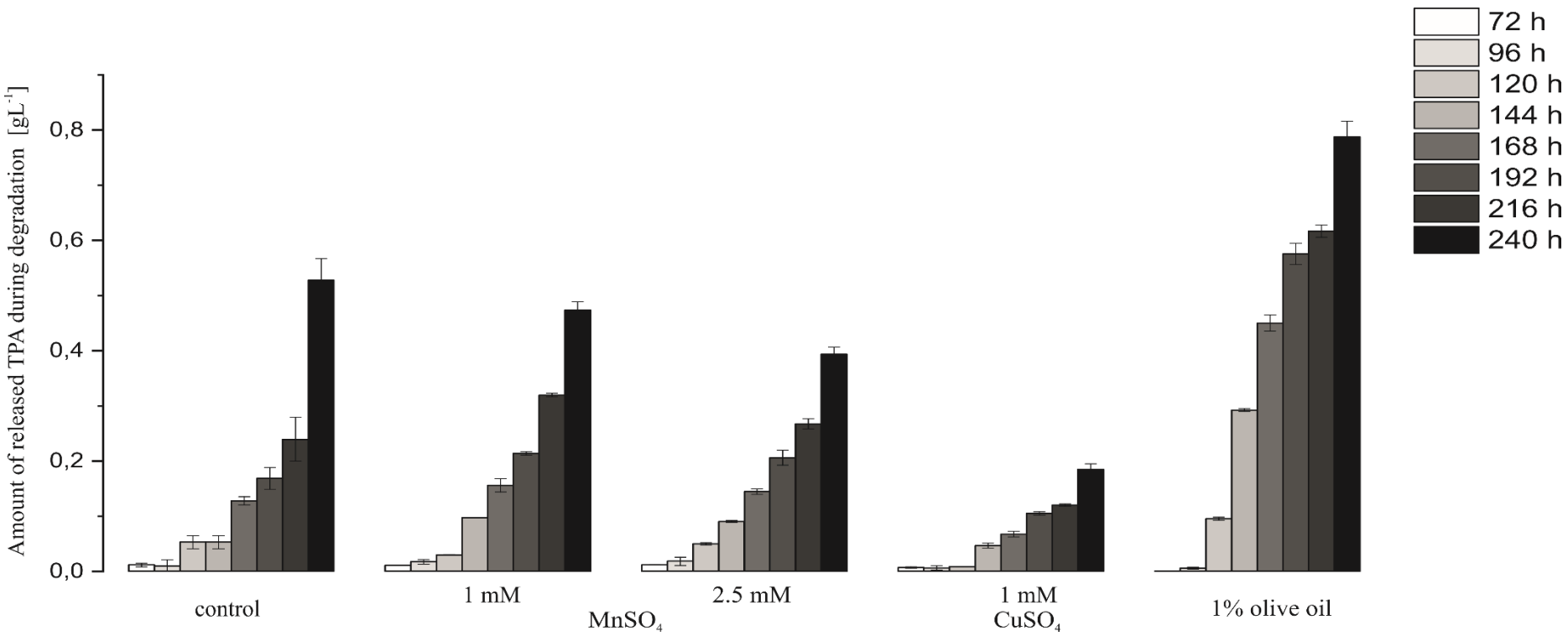
Effect of hydrolysis products and its solvents to *Y. lipolytica* growth.

A. YPD medium containing TPA and DMSO solvent.
B. YPD medium containing MHET and methanol solvent
C. YPD medium with EG.



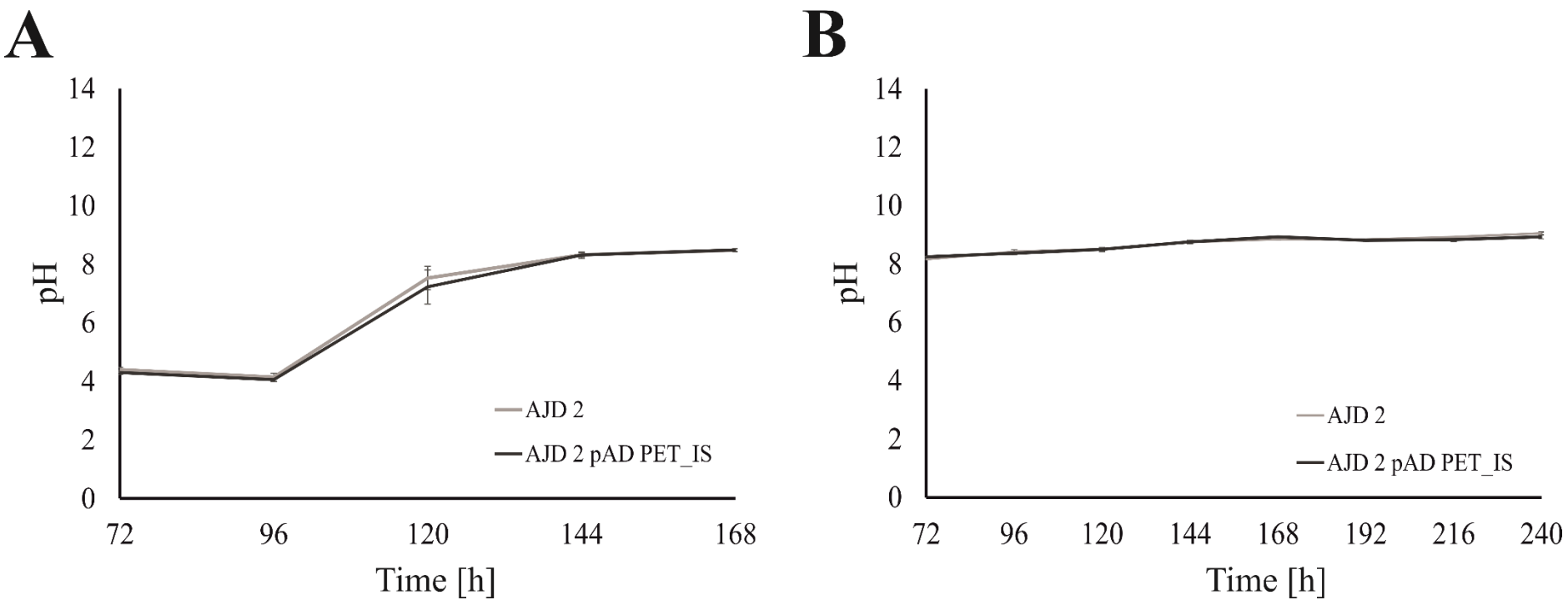
Supplementary Fig 2.

Daily increase of hydrolysis product measured in the supernatants during cultivation of AJD 2 pAD PET_IS in deep well plates.



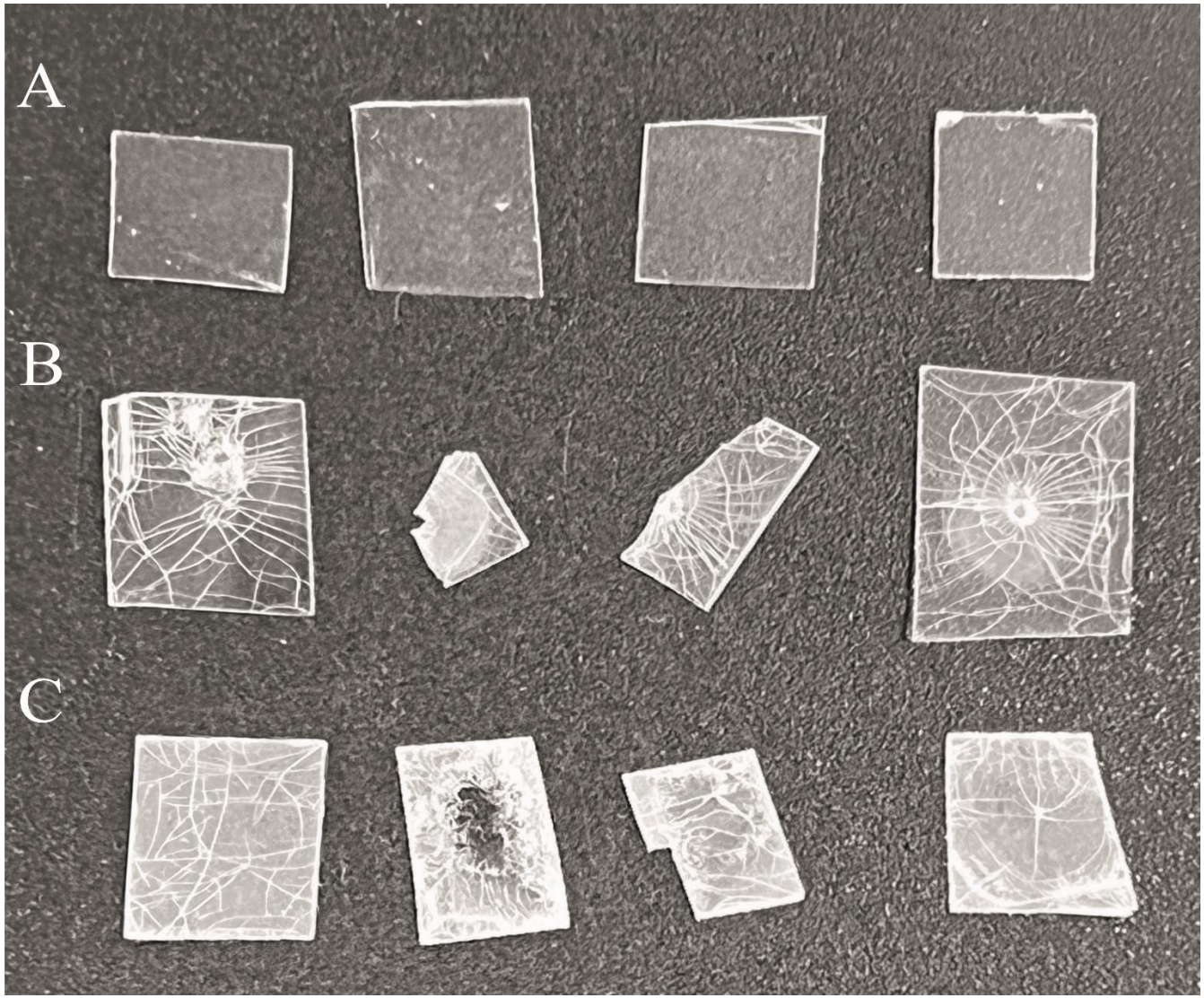
Supplementary Fig. 3

Daily increase of the PET hydrolysis product in AJD 2 pAD PET_IS culture carried out in 0.3-L Erlenmeyer flasks.



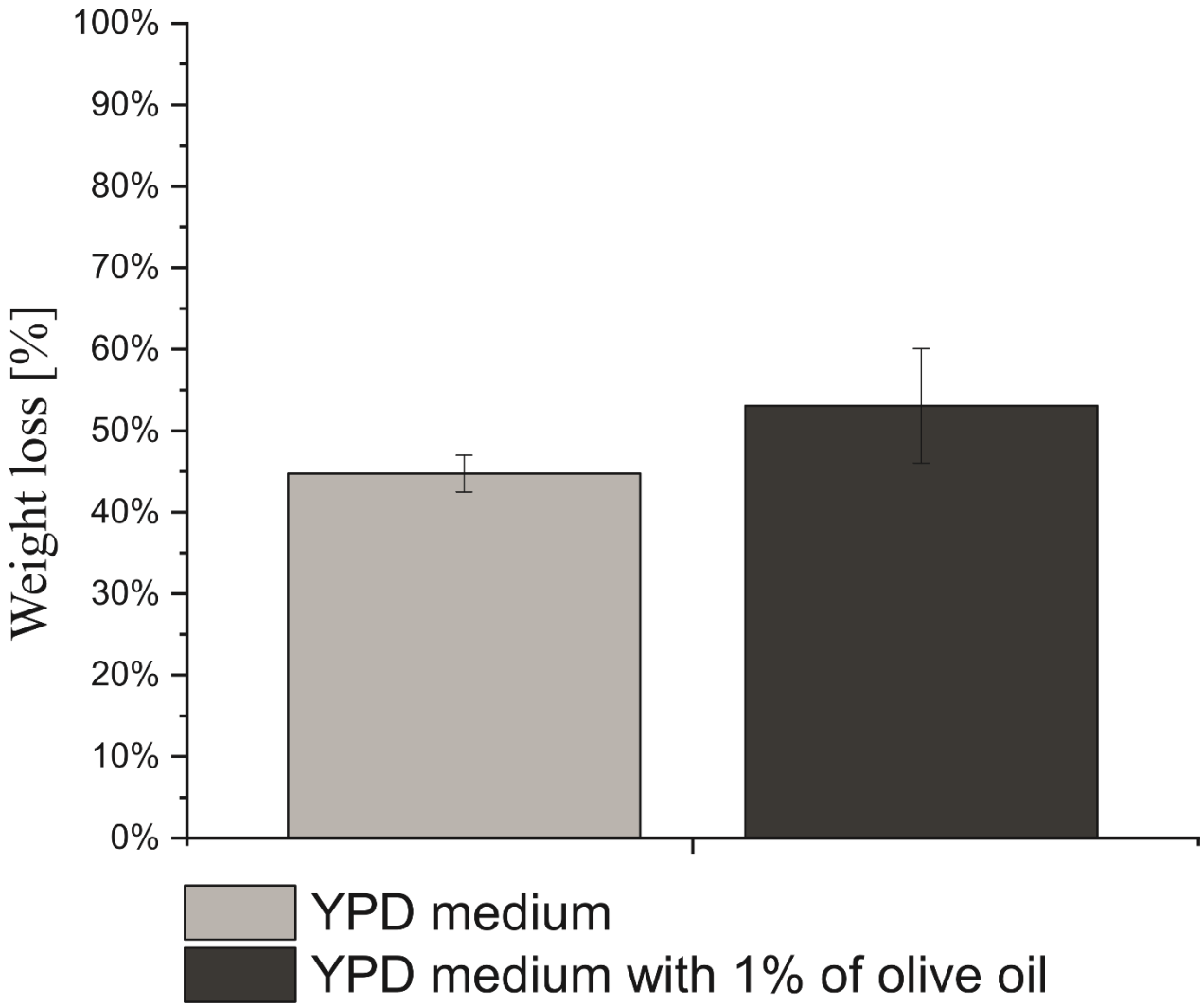
Supplementary Fig. 4

pH changes during AJD 2 and AJD 2 pAD PET_FS culture carried out in DPW (A) and 0.3 L Erlenmeyer shake flasks (B) in YPD medium containing 50 gL^{-1} of glucose and 0.266 g (A) or 2g (B) of PET powder. Error bars represent standard deviation



Supplementary Fig. 5

PET films images derived after culture of AJD 2 (A) in YPD media and AJD 2 pAD PET_IS cultivated in YPD media without (B) or with olive oil supplementation (C).



Supplementary Fig. 6

Estimated weight loss of PET film after long-term cultivation with *Y. lipolytica* AJD 2 pAD PET_IS.

Supplementary Table 1

Comparison of the amount of released PET hydrolysis products by various hydrolases and its mutants.

Host-organism	Enzyme	Substrate	Degradation conditions	Degradation time	Amount of PET degradation products released	References
Yarrowia lipolytica	PETase	Amorphous PET powder	Degradation during cultivation at 28 ºC at 200 RPM in YPD medium	240 h	TPA: 0.53 gL^{-1}	This study
			Degradation during cultivation at 28 ºC at 200 RPM in YPD medium supplemented with olive oil		TPA: 0.88 gL^{-1}	
Ideonella sakaiensis	PETase	PET film	Incubation with 50 nM of the purified enzyme at 30 º at pH 7.0	18 h	TPA: 0.09 mM MHET: 0.2 mM	(Yoshida et al., 2016)
		Highly crystallized PET			TPA: 0.007 mM MHET: 0.012 mM	
Escherichia coli	PETase	PET film	Incubation with 50 nM of the purified enzyme at 30 ºC at pH 9.0	144 h	TPA + MHET: 0.12 mM	(Chen et al., 2021)
			Incubation with 50 nM of the purified enzyme at 40 ºC at pH 9.0		TPA + MHET: 0.13 mM	
Escherichia coli	PETase	PET powder	Incubation with crude enzyme supernatant at 30 ºC at pH 9.0	18 h	TPA: 350 µM MHET: 160 µM	(Shi et al., 2021)
				24 h	TPA:360 µM MHET: 220 µM	
				48h	TPA: 400 µM MHET: 130 µM	

Escherichia coli	PETase	Commercial PET film	Incubation with the purified enzyme at 30 ºC at pH 9.0	72 h	TPA: 2.4 μM MHET: 9.1 μM	(Son et al., 2019)
			Incubation with the purified enzyme at 40 ºC at pH 9.0		TPA: 3.3 μM MHET: 5.4 μM	
Escherichia coli	PETase	PET film	Incubation with 5 μg of the purified enzyme at 30 ºC at pH 8.5	48 h	TPA: 2.5 mM	(Ma et al., 2018)
Chlamydomonas reinhardtii	PETase	PET powder	Incubation of 30 mg PET powder with the cell lysate	4 weeks	TPA: 9.12 mg	(Kim et al., 2020)
Fusarium solani	Cutinase	PET yarn	Incubation with 80 U crude enzyme with 2 gL^{-1} of substrate at 30 °C in pH 7.0	168 h	TPA: 9 μgmL^{-1}	(Nimchua et al., 2007)
Fusarium oxysporum					TPA: 16 μgmL^{-1}	
Thermobifida cellulosilytica Thc_Cut1	Cutinase	PET film	Incubation with 200 μgmL^{-1} of purified enzyme with PET film previously washed in Triton X-100 at 50 °C	48 h	TPA: 260 μM MHET: 20 μM	(Acero et al., 2013)
Thermobifida cellulosilytica Thc_Cut2					TPA: 85 μM MHET: 80 μM	
Humicola insolens	Cutinase	Post-consumer PET	Incubation with 1.0 $mg_{protein}mL^{-1}$ of purified enzyme at 70 °C in pH 7.0	96 h	TPA+MHET+BHET: 129 mM	(Eugenio et al., 2021)

Leaf-brach compost	LCC cutinase	PET film	Incubation of purified enzyme at 70 °C at pH 8.0	24 h	Data not shown	(Sulaiman et al., 2014)
Engineered *Pichia pastoris (Komagataella phaffii)*	LCC cutinase	PET film	Incubation with 1 μM purified enzyme at 50 °C in pH 8.0	48 h	Data not shown	(Shirke et al., 2018)
Microbacterium oleivorans	Synergic action of cutinase and other hydrolases	PET film	Combined treatment of *M. oleivorans* JWG-G2 at 5× 103 μLcm^{-2} and 120 μgmg^{-1} of cutinase from *T. Fusca* at 35 °C with constant agitation of 120 RPM	15 h	TPA: 47 nM MHET: 330 nM	(Yan et al., 2021)
Thermobifida fusca						
Engineered *Escherichia coli*	PETase $^{W159H/F229Y}$	PET tablets from bottle	Incubation with14 $mgmL^{-1}$ of purified engineered protein were compared to native enzyme at 40 °C	24 h	Data not shown	(Meng et al., 2021)
Engineered *Escherichia coli*	PETase	PET film	Incubation with 50 nM of purified PETase protein and its conjugates with various monomers at 40 °C in pH 9.0	4 days	TPA+MHET: 36.7	(Chen et al., 2021)
Conjugates	TBMA[1]-PETase				TPA+MHET: 82.5 μM	
	HEMA[2]-PETase				TPA+MHET: 55.0 μM	
	DMAEMA[3]-PETase				TPA+MHET: 77.5 μM	
	MA[4]-PETase				TPA+MHET: 46.7 μM	

[1] TBMA: tert-Butyl Methacrylate
[2] HEMA: Hydroxyethyl Methacrylate
[3] DMAEMA: 2-(dimethylamino)ethyl Methacrylate
[4] MA: Methacrylic acid

Engineered *Escherichia coli*	DuraPETase[5]	PET film	Incubation with 0.5 $mgmL^{-1}$ of purified engineered enzyme at 37 °C in pH 9.0	10 days	TPA+MHET+BHET: 3.1 mM	(Cui et al., 2021)
Engineered *Escherichia coli*	DuraPETase-4M[6]	PET powder	Incubation with 0.01 $mgmL^{-1}$ of purified enzyme at 60 °C in pH 9.0	96 h	TPA+MHET+BHET: 15.8 mM	(Liu et al., 2022)
		preformed PET film from PET powder			Data not shown	
Engineered *Escherichia coli*	LCC cutinase WCCG[7]	Post-consumer PET waste	Incubation with 1 $mg_{enzyme}g_{PET}^{-1}$ at 72 °C in pH 8.0	10.5 h	Data not shown	(Tournier et al., 2020)
	LCC cutinase ICCG[8]			9.3 h	Maximum productivity: 42.1 $g_{TPA}\ l^{-1}\ h^{-1}$	

[5] DuraPETase: *Is*PETase$^{S214H/I168R/W159H/S188Q/R280A/A180I/G165A/Q119Y/L117F/T140D}$

[6] DuraPETase-4M: DuraPETase$^{N233C/S282C/H214S/S245R}$

[7] WCCG: LCC$^{F243I/D238C/S283C/\ Y127G}$

[8] ICCG: LCC$^{F243W/D238C/\ S283C/Y127G}$