POLITECNICO DI TORINO

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Biodegradazione di bio-poliesteri tramite PET idrolasi

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Sommario

S1. Introduzione

S1.1 Il riciclo della plastica

Oggigiorno il continuo uso di prodotti derivanti dal petrolio causa preoccupazioni ambientali ed etiche sempre maggiori. Le risorse fossili sono limitate e stanno terminando, ma, allo stesso tempo, la richiesta di prodotti chimici e specialmente di plastica aumenta. Una soluzione sempre più affermata è quella di produrre tali sostanze a partire dalla biomassa, definita come "materia organica biodegradabile originata da piante, animali e microorganismi" [3]. Avendo quest'ultima consumato, durante i processi fisiologici degli organismi dai quali deriva, anidride carbonica, l'impronta di carbonio dei materiali da questa derivanti è minore rispetto a quella delle plastiche prodotte a partire da sostanze fossili [1]. Motivo per cui il mercato delle bioplastiche è in continuo incremento.

Purtroppo, questo cambiamento non risolve un altro problema relativo alla natura chimica intrinseca delle plastiche in generale, indipendentemente dalla loro origine. Questi materiali sono pensati per resistere nell'ambiente ed evitare una facile degradazione, il che, però, diventa un problema per il trattamento finale di tali composti che, al momento, possono seguire quattro strade, il cui schema è riportato in figura 1.1 [1]. Principalmente i metodi possono essere chimici, meccanici o termici, ma, spesso, producono come componenti secondari sostanze tossiche, a causa di contaminanti presenti nel materiale di origine. Per questa ragione la plastica è ancora in maggioranza accumulata nelle discariche, dove, ridotta in dimensioni dagli agenti atmosferici, raggiunge scale microscopiche, la cosiddetta "microplastica". Quest'ultima ha un effetto nocivo per animali ed esseri umani, se inalata o ingerita [2]. Ad esempio, è stato dimostrato che le microplastiche inibiscono la crescita delle alghe, il che comporta meno ossigeno prodotto da queste tramite fotosintesi [1]. Inoltre, una volta entrate nel corpo umano, le microplastiche tendono a rilasciare i loro monomeri, così come additivi e tossine, portando a problemi fisiologici, come stress ossidativo e carcinomi. [1].

Dunque, risulta necessario cambiare il trattamento finale di questi materiali, utilizzando altre tecniche, che superino il problema della bassa reattività della plastica e che siano, allo stesso tempo, sostenibili, non utilizzando condizioni operative troppo estreme o prodotti chimici inquinanti. Una possibile soluzione potrebbe essere quella di utilizzare alcuni microorganismi, che basano il loro sostentamento proprio su questo genere di polimeri.

S1.2 Obiettivi del progetto

Questo progetto è parte di una ricerca il cui scopo è indagare le caratteristiche di alcune bioplastiche e, in particolare, copre uno dei possibili trattamenti post-utilizzo, la già citata biodegradazione. In particolare, l'obiettivo sarà quello di testare se alcuni enzimi nella loro versione non modificata, noti per poter catalizzare la degradazione del PET, possano idrolizzare i polimeri in esame.

Dopo aver presentato nel capitolo 2 alcuni concetti teorici riguardo la definizione e il mercato attuale delle bioplastiche, presentandone alcuni esempi, si passerà, nel capitolo 3, ad illustrare lo stato dell'arte riguardante i processi innovativi, e non, di riciclo delle plastiche.

Successivamente l'attenzione sarà portata sulla biodegradazione, attraverso due studi:

1. Nel capitolo 4 un'investigazione teorica riguardante il metodo della biodegradazione, con focus sugli enzimi noti per catalizzare la degradazione del PET sarà proposta. Le strutture catalitiche di queste proteine, i meccanismi di legame con i substrati e alcuni esempi di applicazione saranno analizzati grazie ad alcuni studi precedenti. Inoltre, i polimeri oggetto della ricerca saranno descritti, e un loro possibile meccanismo di degradazione catalizzato da tali enzimi sarà proposto. Infine, alcuni metodi biotecnologici, sui quali sarà basata la parte sperimentale, saranno brevemente esposti nel capitolo 5.
2. Nei capitoli 6 e 7 l'analisi sperimentale studierà i prodotti finali dell'idrolisi enzimatica sulle nuove bioplastiche. Prima di eseguire la reazione, gli enzimi saranno espressi e purificati. A questo punto le bioplastiche saranno incubate, in alcuni casi dopo essere state sottoposte ad un lavaggio, con gli enzimi in diverse concentrazioni. I loro prodotti finali saranno analizzati tramite cromatografia liquida ad alta prestazione (HPLC) e gascromatografia (GC).

S2. Le bioplastiche

S2.1 Definizioni e mercato

La definizione di bioplastica è ambigua e soggetta a diverse interpretazioni, nonostante sia stata inserita nel report tecnico 15392 del CEN (European Committe for Standardization) come "plastica derivata dalla biomassa" [3]. La plastica è definita, in EN ISO 472, come "il materiale che contiene come sostanza principale un polimero ad alto peso molecolare e che, ad un certo passaggio della produzione, può ricevere una forma tramite il flusso" [3].

Stando alla definizione data nell'introduzione, dunque, il termine bioplastica non include l'essere biodegradabile una volta disposto nell'ambiente: questo dipende dalle caratteristiche chimiche del polimero, come un gruppo funzionale reattivo, e dall'esistenza di microorganismi che sfruttano tali materiali come fonte di sostentamento. Tuttavia, ad oggi molti studi continuano ad estendere la definizione di bioplastiche includendovi anche l'essere biodegradabile, rendendo l'argomento complesso.

Ciononostante, risulta interessante considerare che ci sono alcune plastiche biodegradabili che sono originate da sorgenti fossili, come il policaprolattone (PCL) o il polibutilene succinato (PBS), oppure, al contrario, bioplastiche che non sono biodegradabili, come il bio-polietilene, per la loro struttura chimica stabile [3,4]. L'attenzione di questo studio è stata rivolta, però, a quelle bioplastiche anche biodegradabili, la cui struttura chimica, i loro metodi di produzione e le loro applicazioni sono stati brevemente spiegati nei paragrafi da 2.2 a 2.6. Si tratta di acido polilattico (PLA), poliidrossialcanoati (PHA), poliglicolide (PGA) e bio-polietilentereftalato (Bio-PET).

Nonostante l'ambiguità relativa alle definizioni, la produzione di bioplastiche non è un processo nuovo, poiché le prime plastiche furono sintetizzate dalla caseina, una proteina del latte, nel 1530, in un'abbazia benedettina della Baviera [3]. Da quel momento, e soprattutto nel XX secolo, il loro utilizzo divenne sempre maggiore, finchè, quando il prezzo del petrolio divenne accessibile negli anni Venti, non furono sostituite da plastiche di origine fossile [3]. Riguadagnata attenzione negli anni Ottanta, a causa della crescente consapevolezza del problema ambientale, il loro mercato è ora in rapido incremento, con 15.8 milioni di tonnellate di termoplastiche vendute in Germania nel 2010, specialmente per il settore del packaging [3].

S3. Tecniche di degradazione delle bioplastiche

Come già discusso, le bioplastiche seguono lo stesso trattamento di smaltimento delle plastiche tradizionali e la maggioranza viene ancora trasferita in discariche o negli inceneritori. Tuttavia, seguendo un andamento che favorisce la sostenibilità e l'economia circolare, nuovi metodi si stanno imponendo, come si può vedere in figura 3.1, che mostra un sistema volto a riutilizzare i prodotti, dopo averli trattati. In tale schema risulta evidente come, sfruttando nuovi processi, si diminuirebbe la percentuale delle plastiche (e delle bioplastiche) portata nelle discariche.

In particolare, i trattamenti più promettenti sono quello termico, sezione 3.1, specialmente la pirolisi catalitica e non, quello chimico, sezione 3.2, con la tradizionale solvolisi, la dissoluzione/precipitazione, l'upcycling, e i più innovativi metodi meccanochimici, il foto-reforming a temperatura ambiente e la biodegradazione [2]. Poiché questo progetto si focalizza su quest'ultimo trattamento, basato a sua volta su una reazione di idrolisi catalizzata da enzimi, nei prossimi paragrafi verrà fornito un sommario di questi due temi, lasciando l'approfondimento dei restanti processi nelle sezioni già citate.

S3.1 Idrolisi

L'idrolisi si inserisce nel più generico concetto di solvolisi, che indica la completa o parziale depolimerizzazione dei polimeri con o senza un catalizzatore [1]. A seconda del reagente la solvolisi si divide in glicolisi, con inserimento di un glicol [1], alcolisi, che comporta la transesterificazione del poliestere per mezzo di un alcol [1], fosforolisi, ammonolisi e aminolisi [2] e la già citata idrolisi. Quest'ultima prevede la depolimerizzazione per mezzo delle molecole d'acqua, le quali prima diffondono nella matrice del campione e poi reagiscono con i gruppi funzionali reattivi, dando come prodotto finale i monomeri o i dimeri [1]. I due passaggi avvengono simultaneamente, ma, se la diffusione è troppo lenta la reazione avviene solo sulla superficie e, viceversa, se troppo veloce solo nel bulk [1].

Nella sezione 3.2.2 è riportato un modello cinetico dell'idrolisi del PLA, modellato da Gorrasi e Pantani [15]. Tale scelta è stata fatta per mettere in luce come questa reazione sia autocatalitica: una volta formati i primi dimeri con acidi carbossilici come gruppi terminali, essi stessi si comportano da reagente rispetto alla restante parte non ancora idrolizzata [15]. Inoltre, la reazione avviene sempre prima nella parte amorfa del materiale, dove le molecole d'acqua riescono a diffondere più velocemente, e, solo in un secondo momento, in quella cristallina [15]. Tali considerazioni sono utili anche per comprendere il fenomeno della biodegradazione, che si basa comunque sull'idrolisi, per le molecole considerate in questo studio, poiché anch'esse sono poliesteri.

S3.2 Biodegradazione

La biodegradazione è un metodo innovativo di trattamento plastiche che sfrutta la capacità naturale di alcuni microorganismi, evoluta in seguito al continuo incremento di polimeri nell'ambiente, di idrolizzare i legami estere per usare i monomeri come fonte di carbonio [17]. Tale reazione avviene grazie all'azione catalizzante di particolari enzimi, in genere idrolasi, che tali microorganismi producono. Dunque, tale processo può essere condotto incubando tali microorganismi sui polimeri, oppure utilizzandone direttamente gli enzimi.

Il sistema è, dunque, basato su un'idrolisi catalizzata dagli enzimi: la reazione può dunque essere pensata come eterogenea e il suo modello cinetico, riportato nella sezione 3.3.1 e

ricavato anch'esso da Gorrasi e Pantani [15], elaborato tenendo conto sia della degradazione effettiva che della diffusione degli enzimi nel substrato.

Indipendentemente dall'utilizzo diretto degli enzimi o meno, il processo dipende da [16]:

- Condizioni operative (abiotiche, come pH, temperatura e concentrazione, e biotiche, concentrazione degli enzimi, idrofobicità del substrato e surfactanti presenti)
- Caratteristiche del polimero, come flessibilità, grado di cristallinità, morfologia, gruppi funzionali, crosslinking, peso molecolare, copolimeri, additivi.

Tra questi parametri il più impattante rimane la cristallinità, sia per come agisce sull'idrolisi, meccanismo già accennato nel paragrafo precedente, sia perché i microrganismi, e i loro enzimi, sono meno propensi ad agire su un substrato con struttura più organizzata [15].

S4. Degradazione enzimatica

In questo studio la biodegradazione è stata condotta utilizzando l'applicazione diretta di due enzimi: la PETase e la cutinasi, ceppo LCC (leaf and branch compost cutinase, per via dell'origine derivante da un composto di foglie e rami).

S4.1 PETase

La PETase è un enzima appartenente al gruppo delle idrolasi, scoperta nel 2016 dal gruppo di ricerca di Yoshida nel microorganismo *Ideonella sakaiensis.* Esso è capace di catalizzare l'idrolisi del PET, dando, come maggiori prodotti finali, il mono (2-idrossiletil) acido tereftalico (MHET) e, in misura minore, l'acido tereftalico (TPA) e il bis (2-idrossietil)-TPA [17]. Per completare la reazione, producendo i due monomeri costituenti il PET, ovvero l'acido tereftalico e il glicol etilene, un secondo enzima, noto come MHETase e presente nello stesso microorganismo, viene coinvolto [17]. In questa ricerca, tuttavia, essendo MHETase poco conosciuto, ma, soprattutto, essendo estremamente selettivo verso MHET, solo PETase è stata utilizzata nella procedura sperimentale. Informazioni più dettagliate circa struttura meccanismo di MHETase possono essere trovate nel paragrafo 4.3.

La struttura della PETase è costituita da un classico ripiegamento con 8 foglietti β e 6 α eliche, come riportato dallo studio condotto da Austin et al. [17]. Il sito attivo ricorda quello di altre idrolasi ed è composto da una triade catalitica di Ser160, Asp206 e Hist237, posta in un anello, con il primo residuo nel "gomito nucleofilico" in una particolare sequenza detta "consenso" (Gly-Trp159-Ser-X2-Gly) [17]. L'enzima ha una superficie altamente carica e presenta due ponti disolfuro, uno adiacente il sito attivo e l'altro vicino al terminale C della proteina [17].

Come la PETase agisca per catalizzare l'idrolisi del PET non è ancora perfettamente noto e diverse ipotesi sono state avanzate, tre di queste sono descritte nella sezione 4.2.2. Le prime due, di Chen [18] e Joo [19], si basano sul meccanismo dell'adattamento indotto, mentre la terza, di Wei [20] prende in considerazione le interazioni deboli che le unità aromatiche del substrato formano con gli amminoacidi idrofobi. In tutti e tre i casi, comunque, la reazione prevede la formazione di catene polimeriche intermedie e, solo dopo numerosi passaggi di idrolasi, la trasformazione a dimeri e a monomeri.

Nella sezione 4.2.3 sono state proposte alcune modifiche fatte da gruppi di ricerca tra il 2016 e il 2020 all'enzima PETase originario. Tali cambiamenti si sono focalizzati su due direzioni: rendere il sito attivo della PETase più simile a quello delle cutinasi, aumentandone il volume, per renderlo più accessibile a substrati di dimensioni maggiori del PET, e incrementare l'efficacia dell'enzima anche a temperature più alte di quelle ottimali.

S4.2 Cutinasi

La cutinasi è un enzima appartenente alla famiglia delle α/β idrolasi, metabolizzato sia da batteri che da funghi, per permettere a tali organismi di catalizzare la degradazione della cutina delle piante, polimero composto da cere e altri polimeri [24]. L'enzima può anche catalizzare l'idrolisi di altre molecole, come i triacilgliceroli ed esteri solubili dal peso molecolare basso, ma anche catalizzare reazioni di esterificazione e transesterificazione [24].

La struttura di questo enzima è uguale a quella della PETase, con la stessa triade catalitica e la stessa struttura secondaria di β-foglietti e α-eliche [24]. Nonostante queste somiglianze, i due

enzimi mostrano però delle differenze sostanziali, elencate nel paragrafo 4.6. Principalmente la cutinasi e la PETase hanno una superficie diversamente carica: neutra la prima, carica, con un punto isoelettrico di 9.6, la seconda [17]. Tale differenza comporta una diversa selezione di substrati legati, idrofili e carichi per PETase e neutri per cutinasi [17]. Inoltre, la PETase ha un sito attivo che agevola l'entrata di tutti i composti che contengono un anello benzoico, grazie ad un cambio di residui: serina rispetto alla fenilalanina della cutinasi [17]. Tuttavia, la differenza maggiore tra i due enzimi rimane il numero di ponti disolfuro: due nella PETase, solo uno nelle cutinasi, in prossimità del C terminale della proteina [17].

A causa di tali differenze strutturali, la cutinasi sembra essere meno efficiente nei confronti dei poliesteri semi-aromatici, ma, comunque, può catalizzarne l'idrolisi, come riportato nella sezione 4.4.2, dove alcuni studi riportanti tale abilità sono stati descritti. Per aumentare l'efficacia di questo enzima verso substrati come il PET, alcuni studi sono stati brevemente descritti nel paragrafo 4.4.3 e sono tutti basati nel rendere la sua struttura più simile a quella della PETase, per permettere alle molecole aromatiche di potervi accedere.

In questo studio è stata utilizzata la sequenza descritta nel 2012 da Sulamain et al., ovvero quella della LCC. In tal caso, dunque, l'enzima non proviene da un preciso microrganismo, ma è stata trovata nei residui di foglie e rami dopo essere stati trattati con urea [27]. La scelta è ricaduta su tale enzima perché il gruppo di ricerca ha recentemente pubblicato i suoi risultati nella degradazione del PET, anche prevedendo un'analisi della temperatura ottimale di reazione, sulla quale ci si è basati.

S4.3 Bio-poliesteri sperimentali

Le bioplastiche utilizzate in questo studio sono quattro diversi tipi di poliesteri derivanti da biomasse con origini diverse, descritte nel paragrafo 4.7. Dunque, i monomeri costituenti sono acidi organici o dioli, combinati diversamente, ma molto simili tra di loro. Nei primi due polimeri, P1 e P2, tali monomeri sono uniti per policondensazione a formare eteropolimeri alifatici.

In P1, figura S4.1, l'acido maleico, che può essere prodotto tramite ossidazione di zuccheri [28] (denominato A per brevità, figura 4.12) è stato fatto reagire con un diolo proveniente da terpeni (monomero D, figura 4.14). Di quest'ultimo monomero la struttura risulterà parzialmente coperta per motivi di confidenzialità; tuttavia, tale scelta non influirà sulla comprensione dei risultati finali, non essendo direttamente coinvolta nel legame con l'enzima.

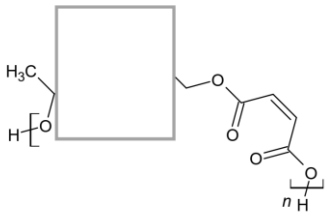

Figura S4. 1 Struttura chimica di P1

P2 differisce da P1 solo per l'acido organico utilizzato, in questo caso acido itaconico (figura 4.13). Quest'ultimo è il prodotto finale del metabolismo di alcuni funghi, tra cui *Aspergillus itaconicus* e *Aspergillus terreus* [29].

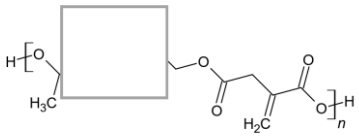

Figura S4. 2 Struttura chimica di P2

Gli altri polimeri, invece, sono copolimeri semi-aromatici che hanno due gruppi principali: il primo è comune a quello di P1 e P2, il secondo presenta, legato a D, un acido organico aromatico, 2,5 acidofurandicarbossilico, FDCA (C, figura 4.17), derivante da disidratazione selettiva di alcuni zuccheri [29].

Il copolimero P3 ha, dunque, in comune con P1 la prima unità ripetitiva e, come seconda, presenta D legato a C, come si può notare dalla figura S4.3.

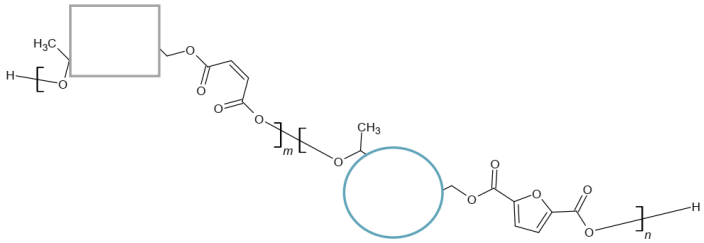

Figura S4. 3 Struttura chimica di P3

Infine, il copolimero P4, simile in parte a P2, condivide la stessa seconda unità ripetitiva con P3.

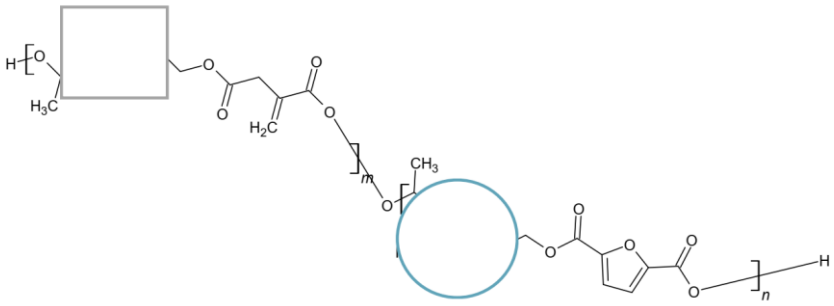

Figura S4. 4 Struttura chimica di P4

Per maggior informazioni circa i meccanismi di polimerizzazione adottati si rimanda al paragrafo 4.7.1 e 4.7.2.

Tutti i polimeri sono stati forniti dopo essere stati sottoposti ad un processo di cross-linking tramite applicazione di raggi UV e utilizzando un iniziatore e agente fotoattivo, il trimetilpropano tris(3-mercaptoproprionato), TMTP, figura 4.21. In figura S4.5 si può osservatore il P2 con i ponti zolfo-carbonio.

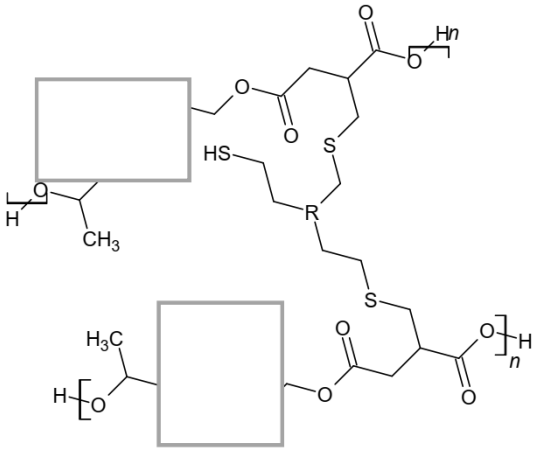

Figura S4. 5 Versione post cross-linking di P2

S4.4 PETase e cutinasi nella degradazione dei bio-poliesteri sperimentali

Nei paragrafi precedenti è stato possibile notare come PETase e cutinasi, pur avendo una struttura simile e la stessa triade catalitica, prediligano substrati diversi, con il primo molto più selettivo rispetto a polimeri semiaromatici. Ciononostante, entrambi possono essere utilizzati nella degradazione di poliesteri.

Come affermato in precedenza, la biodegradazione enzimatica non dipende solo dalle caratteristiche chimiche del polimero, ma anche dalla sua cristallinità, dalla sua idrofilia/idrofobicità, dall'orientamento della sua catena e da altre proprietà morfologiche [34], senza considerare l'enzima stesso, con le sue preferenze in termini di temperatura, medium e altre condizioni operative. Tuttavia, in questo studio si è scelto di basare le

considerazioni solo sulla aromaticità o alifaticità dei polimeri, considerando che gli enzimi adottati non sono stati ingegnerizzati per il substrato.

P1 e P2 sono eteropolimeri alifatici, dunque una loro ampia degradazione tramite PETase non è aspettata, mentre la cutinasi dovrebbe catalizzare l'idrolisi dei loro legami estere, considerando l'ampia gamma di prodotti che è capace di trattare. In ogni caso il meccanismo dovrebbe procedere portando prima alla formazione di dimeri e, solo in un secondo momento, dei monomeri, come è visibile nello schema S4.6 per il polimero P1. Schema simile per P2 può essere trovato nella sezione 4.8.1, figura 4.23.

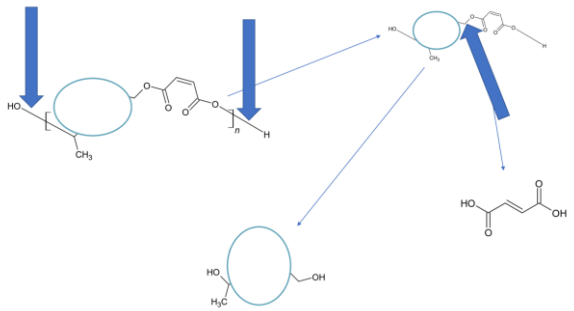

Figura S4. 6 Meccanismo di degradazione di P1

P3 e P4 sono, invece, polimeri aromatici per via della presenza di un furano nel monomero C, FDCA. Per questa ragione la PETase dovrebbe poter accomodare meglio questa struttura nel sito attivo, dando maggiori prodotti rispetto alla cutinasi. Tuttavia, secondo uno studio di Yoshida riportato da Shirke et al. [36], LCC potrebbe dare più risultati anche rispetto a PETase nel trattare questo genere di polimeri, se la temperatura venisse alzata: tale scelta sarà seguita anche in questo studio.

Il meccanismo di degradazione, in ogni caso, sarà per P3 e P4 molto simile a quello di P1 e P2, con la differenza che, essendo copolimeri, in un primo momento l'idrolisi libererà i due dimeri diversi, come si può vedere nella figura S4.7. Anche in questo caso il meccanismo di P4 può essere trovato nella sezione 4.8.2, figura 4.25.

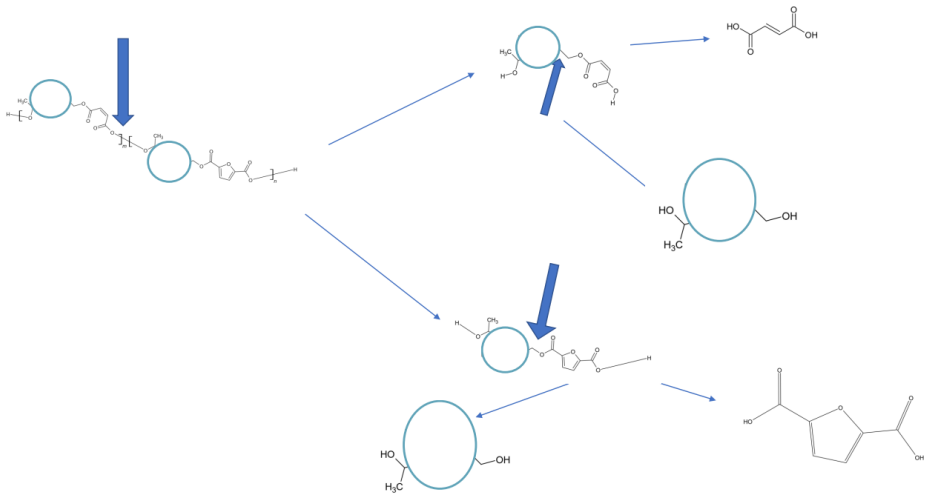

Figura S4. 7 Meccanismo di degradazione di P3

Indipendentemente dalla natura dei polimeri, bisogna ricordare che tutti sono stati forniti nella forma con crosslinking, il che comporta minore cristallinità, ma anche maggiore rigidità [37], con conseguente difficoltà per questi ultimi ad entrare nel sito attivo. Nella sezione 4.8.3 è riportato uno studio di Edwards et al. [37], nel quale sono presentate diverse modalità per devulcanizzare, ovvero rompere i ponti disolfuro, i substrati, rendendoli più facilmente accessibile all'enzima. Tra queste le più importanti sarebbero innalzare la temperatura fino a 275 °C e applicare il dipropil disulfide (DPDS) [37]. Il range di temperatura non è compatibile con gli enzimi, specialmente non essendo stati ingegnerizzati, quindi la scelta di utilizzare dei prodotti chimici si dovrebbe rivelare l'unica possibile. Tuttavia, per rendere il processo completamente sostenibile, un'analisi accurata dovrebbe essere svolta nel futuro. Per quanto riguarda questo studio ci si è limitati ad osservare la degradazione applicando solo gli enzimi e tenendo conto dei crosslink nel caso di prodotti finali in concentrazioni basse o con pesi molecolari diversi rispetto ai monomeri o ai dimeri aspettati.

S5. Risultati sperimentali della degradazione per mezzo di enzimi

In questo paragrafo sono riportati i principali risultati della ricerca sperimentale, ovvero i cromatogrammi risultanti da cromatografia liquida e gascromatografia effettuati sulla soluzione tampone dove i polimeri sono stati immersi per 24 e 48 ore con diverse concentrazioni di enzima (0 µg enzima per avere un controllo, 1 µg enzima/5 mg polimero, 15 µg enzima/5 mg polimero). La procedura sperimentale, comprendente materiali e metodi usati, anche per la produzione degli enzimi, può essere trovata nel capitolo 6.

I risultati preliminari, ovvero quelli riguardanti la produzione in laboratorio di enzimi tramite costruzione di plasmidi e i cromatogrammi dei singoli monomeri sono disponibili nelle sezioni 7.1 e 7.2. Di questi ultimi è necessario sottolineare che i loro caratteristici tempi di ritenzione sono minori di 1 minuto.

S5.1 Applicazione della PETase

I polimeri sono stati sottoposti all'azione della PETase a 30°C dopo aver subito diversi trattamenti, come riportato nella sezione 6.2.2. I risultati migliori, come visibile nella tabella S5.1, sono stati trovati per P3 e P4 dopo 48 ore, con una concentrazione di PETase di 1 µg/ 5 mg di plastica, dopo essere stati lavati con diversi detergenti per rimuovere proteine e cariche superficiali, che avrebbero potuto disturbare l'azione dell'enzima.

Tabella S5. 1 Tempi di ritenzione di prodotti di degradazione per i diversi polimeri sottoposti a differenti concentrazioni di enzima dopo 48h di incubazione

	Tempo di ritenzione (min)			
	P1	P2	P3	P4
1 µg PETase per 5 mg polimero	/	2.344	2.344 2.997 3.308	2.338 2.990
15 µg PETase per 5 mg polimero	2.336 2.989	2.346	/	/

Nella figura S5.1 è visibile il cromatogramma di P3, risolto nello spettro UV a 230 nm.

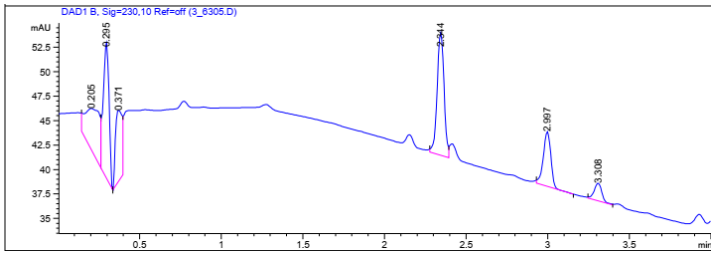

Figura S5. 1 Cromatogramma di P3, lavato, dopo essere stato incubato 48h con 1µg di PETase/5 mg polimero

I tempi di ritenzione sono molto elevati e suggeriscono che i prodotti finali siano dimeri, i più apolari dei singoli monomeri e per questo ritenuti maggiormente in una colonna con base fenilica. Il risultato non sorprende, ricordando che, anche nel caso della degradazione del PET questo enzima è utilizzato nella prima fase dell'idrolisi, quella che comporta proprio la produzione di dimeri.

Tale intuizione è poi stata confermata dai risultati di cromatografia liquida e gascromatografia utilizzando uno spettroscopio di massa, figure S5.2 e S5.3.

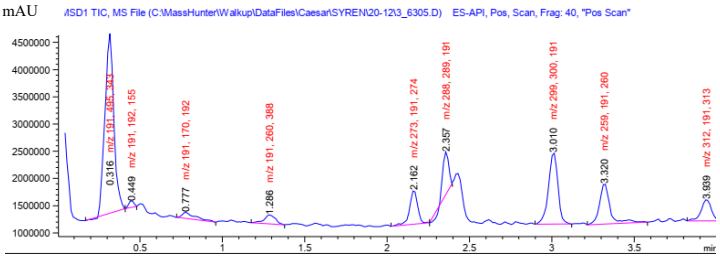

Figura S5. 2 Cromatogramma di P3, lavato, dopo essere stato incubato 48h con 1µg di PETase/5 mg polimero, identificato tramite spettroscopio di massa

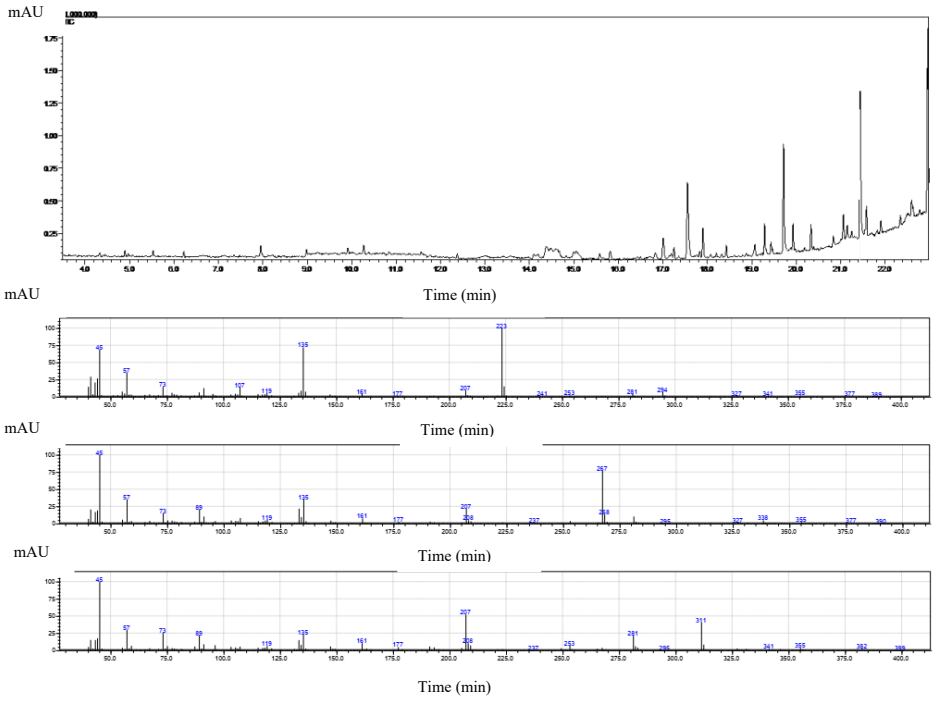

Figura S5. 3 Cromatogramma di GC di P3, lavato, dopo essere stato incubato 48h con 1µg di PETase/5 mg polimero, identificato tramite spettroscopio di massa

Considerando i risultati della cromatografia liquida, i pesi molecolari si trovano in un range che va da 260 g/mol a 313 g/mol. I dimeri di P3 hanno pesi molecolari di 272 g/mol e 310 g/mol. Poiché i risultati della spettroscopia di massa non sono sempre esatti e, tenuto presente i crosslink che avrebbero potuto far variare i pesi, si può dire, con una certa approssimazione, che i prodotti rilasciati siano effettivamente dimeri.

La gascromatografia, svolta per confermare i risultati, ha fornito picchi con pesi molecolari molto simili, 267 g/mol e 311 g/mol, ma anche altri di peso minore, che, nonostante possano rappresentare altri oligomeri, è probabile che siano dovuti alla soluzione tampone stessa.

I risultati della degradazione del polimero P4 sono meno evidenti, ma forniscono le stesse indicazioni: picchi ad elevati tempi di ritenzione, con pesi molecolari compresi tra 200 e 310 g/mol, figure 7.21, HPLC, e 7.37, GC.

Benché questo studio non avesse come obiettivo la produzione di dati quantitativi, come una resa o una conversione di processo, è interessante confrontare la quantità dei prodotti di degradazione di P3 e P4 aventi simili tempi di ritenzione nella HPLC. Per fare ciò, non avendo a disposizione dati circa i dimeri puri, è stato scelto di utilizzare una retta di calibrazione dei monomeri, quella più precisa, ovvero derivante dalle misurazioni effettuate sul monomero B, equazione 7.1. Il grafico di figura S5.4 mostra tale comparazione. Appare evidente come P3 sia stato degradato maggiormente rispetto a P4 in entrambi i casi: l'acido maleico, monomero A, potrebbe essere stato inserito più facilmente nel sito attivo rispetto all'acido itaconico, monomero B.

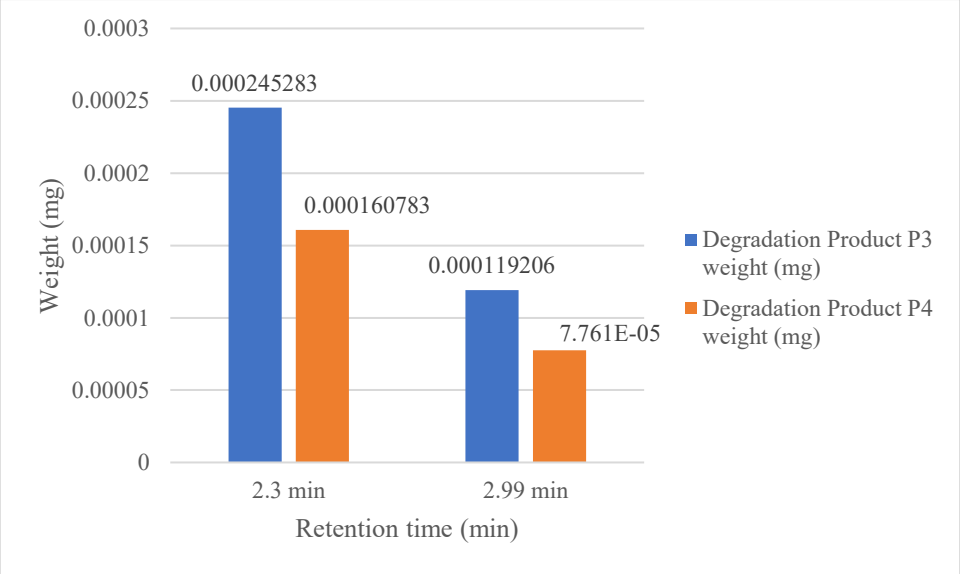

Figura S5. 4 Comparazione quantitativa dei prodotti di degradazione di P3 e P4

In ultima analisi sono state ricavate le immagini dal microscopio elettronico a scansione (SEM) delle superfici dei polimeri post degradazione, disponibili nella sezione 7.5. Purtroppo, queste non forniscono i risultati aspettati: sono presenti buchi sia nei polimeri dove l'enzima è stato applicato, sia nei controlli. Poiché, comunque, i cromatogrammi in tali casi mostrano risultati diversi, sembra possibile che la degradazione sia avvenuta in altra parte del polimero, non sulla superficie. Nell'analisi post applicazione di LCC le immagini SEM non sono, perciò, state effettuate.

S5.2 Applicazione della LCC

LCC è stata applicata sui polimeri per 48 h a 60°C, dopo aver effettuato una misurazione relativa alla temperatura di denaturazione della proteina e aver accertato che avvenisse oltre

tale valore (80°C). I risultati, riportati in tabella S5.2, mostrano una maggiore efficacia dell'enzima rispetto a PETase: tutti i polimeri hanno rilasciato componenti di degradazione, anche con tempi di ritenzione minore di 1 minuto, quindi comparabili con i cromatogrammi dei monomeri.

Tabella S5. 2 Tempi di ritenzione di prodotti di degradazione per i diversi polimeri sottoposti a differenti concentrazioni di enzima dopo 48h di incubazione

	Tempo di ritenzione (min)			
	P1	P2	P3	P4
1 µg LCC per 5 mg polimero	0.441 0.528 0.948 3.053	0.444 0.533 3.058	0.445 0.533 2.663	0.445 0.534 2.021
15 µg LCC per 5 mg polimero	0.443 0.752 2.521	0.441	0.444 2.660	2.017

Nella figura S5.5 è riportato il cromatogramma di HPLC risolto nello spettro UV a 230 nm per il polimero P1 senza applicazione di enzima, dopo essere stato incubato a 60°C per 48h. Appare evidente come la sola applicazione di una temperatura alta abbia prodotto dei dimeri, con tempi di ritenzione intorno ai 2 min e in parte comparabili con quelli esaminati nel paragrafo precedente. Tuttavia, guardando allo spettro ricavato dallo spettroscopio di massa, si può notare come le intensità di tali picchi siano basse e, talvolta, neanche identificabili e il loro peso molecolare non sia comparabile rispetto al dimero di P1. Sembra dunque probabile che si tratti di prodotti di una degradazione termica, che non ha proceduto secondo meccanismi precisi come quella enzimatica.

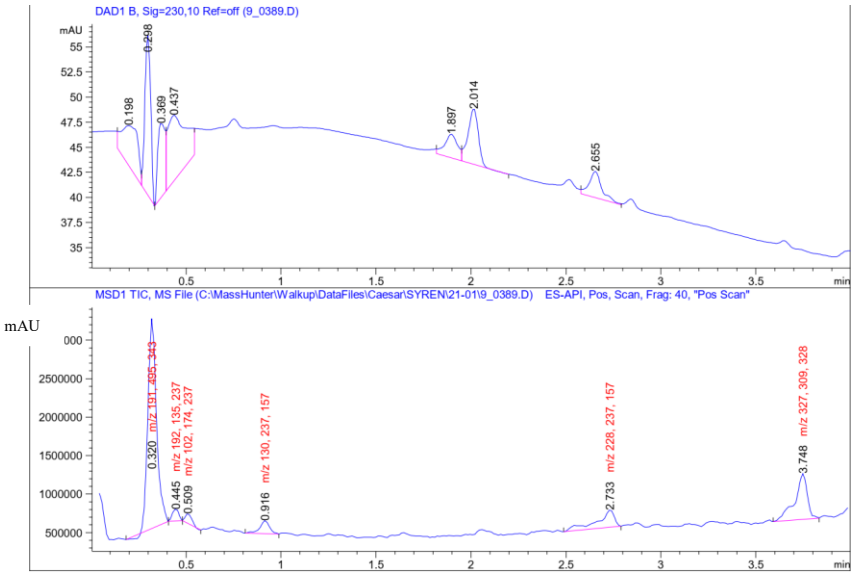

Figura S5. 5 Cromatogramma di P1, lavato, dopo essere stato incubato 48h con 0 µg di LCC/5 mg polimero a 60°C

La figura S5.6 è invece il cromatogramma dei prodotti di degradazione del polimero P1 trattato con 1 µg LCC/5 mg polimero. Il picco a 0.441 min, di grande intensità, suggerisce una degradazione proseguita oltre la formazione di dimeri. Confrontando tale cromatogramma con quello dei monomeri dispersi nella soluzione tampone si nota una somiglianza con quello del monomero D, il diolo, figura 7.6. Hanno lo stesso tempo di ritenzione e presentano un peso molecolare uguale, 147 g/mol, nello spettro MS. Sfortunatamente gli altri monomeri non sono osservabili, anche se la loro formazione contemporanea a quella del monomero D è ritenuta probabile.

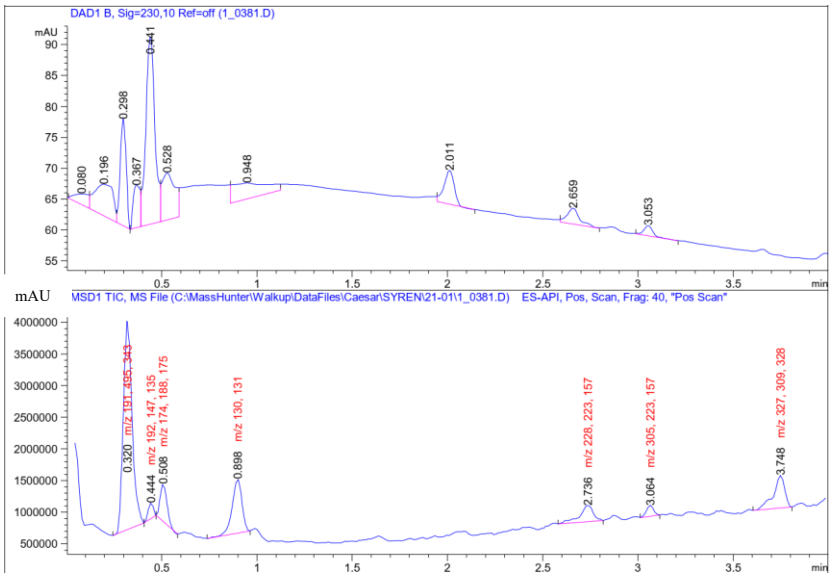

Figura S5. 6 Cromatogramma di P1, lavato, dopo essere stato incubato 48h con 1 µg di LCC/5 mg polimero a 60°C

Gli altri polimeri hanno fornito risultati simili a quelli di P1 e possono essere trovati nella sezione 7.6, tutti presentano picchi ad un tempo di ritenzione intorno 0.400 min, anche se con minore intensità. In figura S5.7 sono indicati i risultati quantitativi di produzione del monomero D in tutti e quattro i casi, dopo 24h e 48h, grazie all'uso della retta di calibrazione di D, avente equazione y=2288.9x.

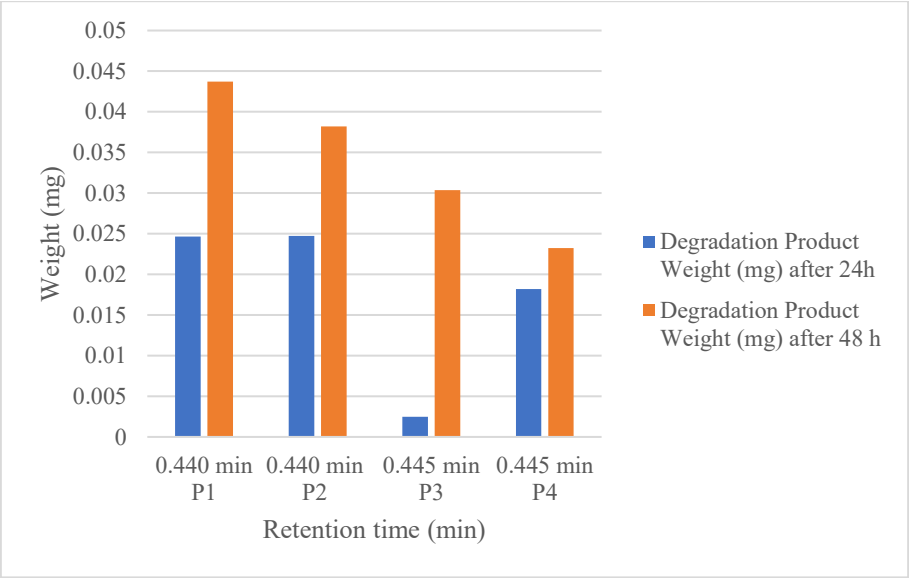

Figura S5. 7 Comparazione quantitativa dei prodotti di degradazione dei quattro polimeri dopo 24 e 48 ore

I due eteropolimeri alifatici, P1 e P2, diversamente da quanto accaduto con l'applicazione della PETase, mostrano maggiori risultati, probabilmente a causa di una minore complessità della loro struttura. Non avendo LCC selettività solo verso i composti aromatici come PETase, tale minore complessità si è tradotta in una maggiore efficienza, anche se probabilmente la degradazione di per sé è stata aiutata dalla temperatura più alta. Importante segnalare, infine, che anche in questo caso i polimeri aventi l'acido maleico anziché acido itaconico, ovvero P1 e P3, sono stati degradati in maggiore quantità: tale cambiamento è dovuto probabilmente ad una più semplice relazione con il sito attivo dell'enzima, come per la PETase.

S6. Conclusioni e lavoro futuro

Questo studio ha confermato che impiegare enzimi nella degradazione di poliesteri è una possibilità da tenere in considerazione nella gamma di nuovi metodi di riciclo di tali polimeri. Lo studio teorico ha suggerito un possibile meccanismo catalitico della idrolisi, comparando le caratteristiche dei substrati a quelli di altre bioplastiche, particolarmente PET per la degradazione enzimatica e PLA per il modello cinetico. Soprattutto l'analisi di altri studi riportanti la degradazione di poliesteri tramite utilizzo di PETase e LCC ha permesso di capire che il primo enzima preferisce substrati aromatici e ha come prodotti finali i dimeri, mentre LCC è meno selettivo.

I risultati sperimentali hanno confermato tali ipotesi, specialmente in relazione a PETase, dando la maggiore quantità di prodotti di degradazione per i composti semi-aromatici e solo picchi bassi per quelli alifatici. Inoltre, tali composti sono comparsi tutti a tempi di ritenzione elevati, dando impressione di essere più idrofobi e con strutture più complesse, probabilmente dimeri, rispetto ai singoli monomeri; risultato poi confermato dai pesi molecolari tramite GC-MS. Infine, è stato scoperto, confrontando i risultati dell'applicazione della PETase con e senza pretrattamento, che i polimeri devono essere lavati prima dell'applicazione dell'enzima, per facilitare la formazione dei legami con il sito attivo, rimuovendo eventuali proteine e cariche presenti sulla superficie polimerica.

La cutinasi, invece, è stata non solo più efficace, ma ha anche fornito risultati più omogenei: tutti i polimeri sono stati degradati e un monomero, D, è stato prodotto in ogni caso. Anche se l'elevata temperatura della reazione potrebbe aver aiutato, la differenza con il cromatogramma del controllo suggerisce che la formazione del monomero D sia dovuta solo all'attività dell'enzima. Per poter avere un confronto tra PETase e LCC ancora più efficace in futuro, una variante più termostabile del primo enzima, ad esempio DuraPETase, descritta nel paragrafo 4.2.3, potrebbe essere utilizzata anch'essa a 60°C.

In entrambi i casi sono comunque state osservati alcuni meccanismi comuni. Innanzitutto, bisogna ricordare che i polimeri presentavano tutti i crosslink: anche se questo li ha resi amorfi e ha dunque facilitato sia l'idrolisi che l'applicazione degli enzimi, PETase e LCC non possono rompere legami carbonio-zolfo. Nonostante ciò, probabilmente questa caratteristica ha diminuito la resa finale e fornito pesi molecolari dei prodotti di degradazione diversi da quelli dei dimeri o dei monomeri stessi. Tale problema potrebbe essere superato in futuro rompendo tali legami durante un pretrattamento, applicando temperature maggiori o, più probabilmente, utilizzando composti chimici, quali, ad esempio, detergenti.

Inoltre, tutti i risultati migliori sono stati forniti in seguito all'applicazione della concentrazione più bassa di enzima, mentre la più alta non è stata in grado di modificare sostanzialmente il comportamento già visto nei controlli. Presumibilmente tale comportamento va ricercato nell'azione competitiva degli enzimi verso il substrato, presente in quantità troppo bassa.

I risultati, comunque, hanno mostrato che questi enzimi possono degradare anche altri substrati oltre al PET, il che apre alla possibilità di usarli in applicazioni future, producendo monomeri e oligomeri da immettere nell'economia circolare delle plastiche.

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Biodegradation of bio-based polyesters by PET hydrolytic enzymes

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TRITA 007

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Abstract

In everyday life the consumption of plastic is steadily increasing, arising some concerns not only regarding the origin of these materials, which is generally fossil-based, but also for their end-of-life treatment. If a solution for the first aspect may be found in the utilization of biobased plastics, the other matter is still under investigation. Recycling plastic is a complex process, due to the broad range of polymers used, but also for the presence of contaminants and additives; for these reasons, these substrates are sent, in almost their totality, to landfills or burnt in incinerations, increasing the carbon dioxide content in the atmosphere and producing microplastics. Nowadays some innovative treatments, aimed in improving a circular economy, have been proposed. Among those, employing plastic-growing microorganisms and their hydrolysing enzymes seems a useful tool and it is the object of this study. This research focuses on a possible degradation via enzymatic hydrolysis of four crosslinked bio-polyesters, two which are semi-aromatic and the remaining aliphatic, performing a theorical and an experimental analysis.

Two esterases, PETase and cutinase, are responsible of the degradation: the theorical investigation brought attention on them, which, based on some previous studies, could hydrolase the bonds of polyethylene terephthalate (PET). Results of former research were used to describe their proposed structures and the followed mechanisms. Then, comparing the chemical structure of the substrates and of PET, a possible reaction mechanism was here suggested. This result was tested through the experimental analysis, after having expressed and purified the enzymes. The washed bio-polyesters were then incubated with those proteins in two concentrations for 48 hours and then the degradation products were analysed with liquid and, some in cases, gas chromatography techniques. Samples where PETase was involved were also investigated using scanning electron microscopy (SEM).

PETase could degrade only semi-aromatic polyesters and the products were mainly dimers, as the gas chromatography mass spectrometer confirmed. This was expected since the same result was obtained in other studies. Furthermore, this behaviour occurred only when the samples were washed before the reaction, since their surface was easier to access for the active site. On the other hand, cutinase continued the degradation until reaching the formation of one monomer, the diol, and, generally, the reaction was successful for every substrate and released a higher quantity of dimers. This was explainable not only thanks to a lower selectivity of the enzyme, but also for the higher operational temperature applied, which came from a major thermostability of cutinase compared to PETase. Moreover, it was observed that in both cases a high concentration of the enzymes blocked the enzymatic hydrolysis, due to the competitivity of the protein's active sites. Unfortunately, the SEM images did not give any concluding result, not showing high differences between polymers surfaces treated with the enzymes and the ones that were just incubated without the proteins.

In conclusion, the type and the quantity of the released products confirmed that PETase and cutinase can be applied in degrading not only PET, but also other polyesters. That considered, some future work, mainly aimed in applying a more thermostable PETase and in treating the crosslinks before the incubation, can still be done, before scaling-up the process and substitute the already followed chemical methods applying non sustainable chemicals.

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1. Introduction

Nowadays the use of petroleum-based products is one of the most discussed matters in terms of concerns about environment and ethics. Fossil sources are limited and going towards the depletion, but the world requires chemicals, and especially plastic, in everyday life, so the solution may be to shift to bio-based products. Biomass consumes carbon dioxide, so plastics coming from these materials have less carbon footprint compared to the petroleum-origin ones [1]. This is the reason why bioplastic market is steadily increasing.

Unfortunately, this change does not solve another issue related to the chemical characteristics of plastic, thus always present independently on their origin. Plastic is produced to resist in the environment, preventing an easy degradation, and, moreover, they may contain additives and contaminants, which could be toxic. Hence, when it comes to its end-of-life treatment, the matter becomes complicated and plastic material is still mainly accumulated in landfills, where it is reduced in size by weather conditions, until it reaches microscale dimensions and it becomes the so-called microplastics, which effect on animals and humans may be dangerous, since they can be easily inhaled or ingested [2]. For example, it was shown that microplastics decrease the growth of algae, which leads to less oxygen produced by them via photosynthesis [1]. Moreover, if microplastics enter the body, they release their components, additives and toxins, leading to physiological problems, like oxidative stress and carcinogens [1].

To avoid these risks and to implement the circular economy, plastic is subjected to several treatments, as it can be seen in figure 1.1, aimed in reusing and recycling as much as possible the forming units and the products themselves.

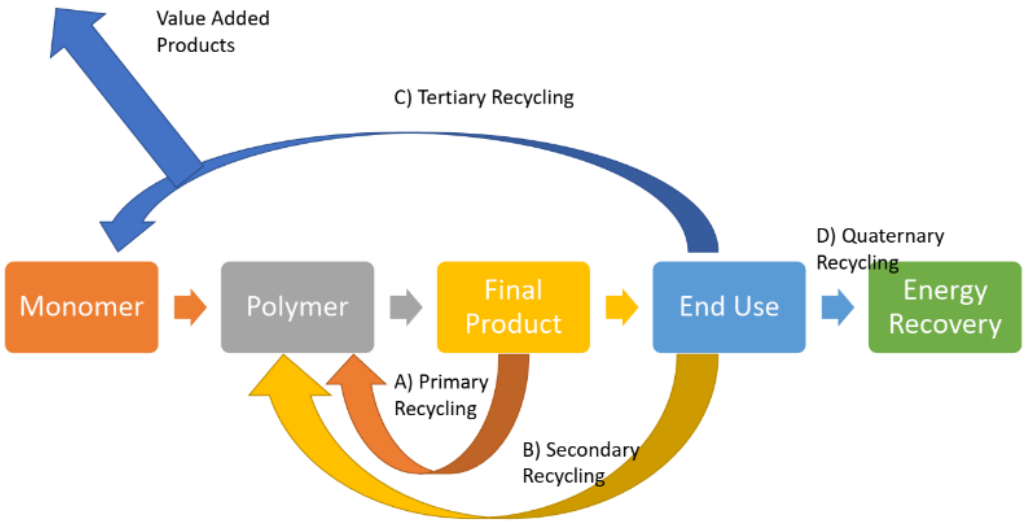


Figure 1. 1 Plastic Recycling options from [1] with modifications

The pathways are divided in:

A. Primary recycling: the polymer is directly reused or simply recycled in a mechanical way, thus preserving its characteristics; it can be done just for plastics of known composition and high quality [1].
B. Secondary recycling: the end use polymer is mechanically recycled into different materials, less complicated structurally [1].
C. Tertiary recycling: the material is chemically recycled; the polymer is broken down into the constituent monomers, which can be then assembled into something else [1].
D. Quaternary recycling: is the incineration of low-grade end use materials, generally waste, to recover the energy, producing, on the other hand, carbon dioxide during the burning [1].

Tertiary recycling involves the use of chemicals, to break down the structure of the polymer; however, this can be achieved also in other ways, using less extreme operative conditions, and avoiding new wastes production from reagents or solvents. Among those, the exploit of some plastic-growing microorganisms is increasing in interest and is currently subject of many researches, especially through the focus on their polymer-degrading enzymes.

1.1 Aim of the project

This project is part of a bigger research aimed in understanding the behaviour of new bioplastics and it covers one of the possible end-of-life treatment methods, the already quoted biodegradation. In particular, the aim will be to investigate whether some enzymes in their wild-type version, known to be able to degrade some plastics, can hydrolyse these involved polymers.

After the presentation of the theorical background of bioplastics definitions and market, with some examples, the state of the art concerning the plastic recycling processes already used, or that will be applied soon, will be explained.

Then, the research will be based on two objectives:

1. A theorical investigation about the biodegradation method, with a focus on enzymes known to be able to disrupt the structure of polyethylene terephthalate (PET) will be proposed. Their catalytic structures, the followed mechanism and some examples will be investigated thanks to some previous studies. The molecules involved in the research will be described, and a possible enzymatic hydrolysis pathway will be suggested. Finally, biotechnological tools, that will be used in the experimental part, will be briefly bring forward.
2. An experimental analysis will study the final products of the enzymatic hydrolysis on new bioplastics. Before running the reaction, the enzymes will be expressed and purified. Then the bioplastics will be submitted to the studied enzymes in different concentrations, for some of the samples after undergoing a washing treatment. Their final products will be analysed through high performance liquid chromatography (HPLC) and gas chromatography (GC).

2. Theorical background: bioplastics

2.1 Definitions and market

Bioplastics are defined, in the Technical Report 15392 of the Technical Committee CEN/TC 249 of the European Committee for Standardization (CEN), as "plastics derived from biomass" [3]. **Plastic** is a broad term, which is, anyway, defined in EN ISO 472, as "materials that contain as an essential ingredient a high polymer and which at some stage of the production can be shaped by flow" [3]; while **biomass** is considered the "non-fossilized and biodegradable organic material originating from plants, animals and micro-organisms" [3].

This definition does not imply, thus, that every plastic coming from biomass is biodegradable if disposed in the environment: just if their decomposition rate is high enough, they are defined in that way. This process happens thanks to microorganisms, in aerobic on anaerobic conditions, which can degrade the polymers and use the energy coming from this break down to growth [3]. To be defined as biodegradable, numerous tests have been proposed: they differ in the process criteria adopted, such as temperature and time [3]. Hence, to be biodegradable, the polymers should have a reactant structure and the biological source does not make any difference. However, sometimes the term bioplastic is also used to mean biodegradable, even if the source is not biomass [4]. This is shown in figure 2.1, where bioplastics can be defined as either biodegradable or biobased. Even though the matter is ambiguous, it is interesting to consider that there are some biodegradable plastics that are coming from petroleum sources, such as polycaprolactone (PCL) and polybutylene succinate (PBS), but there are also bioplastics that are not biodegradable enough, like bio-polyethylene (PE), since their chemical structure is stable and difficult to break [3,4]. And, of course, there are polymers that are both, such as Polylactic acid (PLA) and starch [4]. Acetyl cellulose (AcC) is an exception, since its biodegradability depends on the degree of acetylation: the lower it is, the faster the decomposition [4].

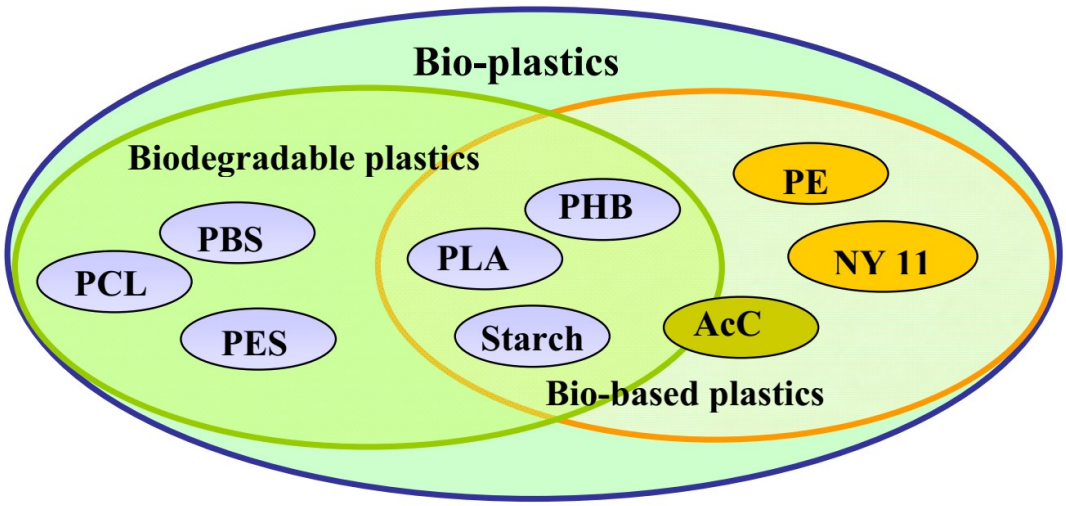


Figure 2. 1 Graph dividing bioplastics in biodegradable and bio-based types [4]

Siracusa and Blanco followed the second definition, bioplastics can be either biodegradable or bio-based or both, and thus proposed a three types-division [5]:

1. Bioplastics both based on renewable sources and biodegradable, such as starch plastics, cellulose polymers, proteins, lignin and chitosan plastics, PLA, PHAs [5].
2. Bioplastics derived from petroleum but still biodegradable, like polycaprolactone (PCL), polybutylene succinate (PBS), polybutylene adipate (PBA) [5].
3. Bioplastics produced from monomers coming both from petroleum sources and biomass [5].

Besides these issues, plastic materials can be also defined as **biocompatible**: they are materials that "don't harm the body or its metabolism", thus are suitable to be used in medical sector [3]. Finally, **biopolymers** are "polymers synthesized by living organisms", including a vast amount of different types of molecules, so it is not a synonym of bioplastics [3]. Moreover, bio-based plastics are not always constituted of biopolymers, such as polylactic acid, which is produced by chemical polymerization of the bio-based monomer, lactic acid [3].

If the definitions concerning bioplastics could be ambiguous, their usage and production is not, and it has been known since the XVI century. The first plastics, mainly thermosets, elastomers, and some thermoplastics, were produced from biomass and not from petroleum sources [3]. The first mention was in 1530: a Bavarian Benedictine abbey produced artificial horn from casein, a milk protein [3]. Other materials then appaired through the century, but it was in 1868, when J. W. Hyatt created the first thermoplastic material, called celluloid from cellulose nitrate and camphor, that plastic industry was created [3]. Bioplastic were then substituted with petroleum derivativities in the 1920s, when crude oil became available at low prices [3]. They gained interest again in the 1980s, when the awareness of the environmental problem raised [3]. Since then their market grew, reaching almost 40% in elastomers and fibres sector in 2010 in Germany, and 15.8 million t for thermoplastics and thermosets, especially for packaging applications [3].

Among the bioplastics that are also biodegradable, the most used and produced are polylactic acid, polyhydroxyalkanoates and polyglycolic acid, as well as bio-Polyethylene terephthalate, bioplastic version of the petroleum-based one. Before studying the chemical composition of the bioplastics that will be used in this research, it seems useful to provide an overview regarding characteristic and industrial production of these well-known materials, to be able to provide a full comparison later.

These molecules are polyesters: their biodegradability comes from the reactant ester group and makes them perfect candidates to solve the accumulation issue of plastics.

2.2 PLA

Polylactic acid (PLA), figure 2.2, is a polymer belonging to the group of aliphatic polyesters composed by α-hydroxyl acids [6].

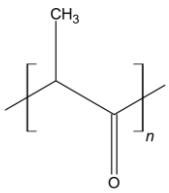


Figure 2. 2 PLA Chemical structure

Its monomeric unit, lactic acid (figure 2.3), chemically called 2-hydroxy-propionic acid, can be found as two chiral stereoisomers, *L* (-) and *D* (+), with the first the most present naturally and a racemic mixture the result of chemically synthetized one [6].

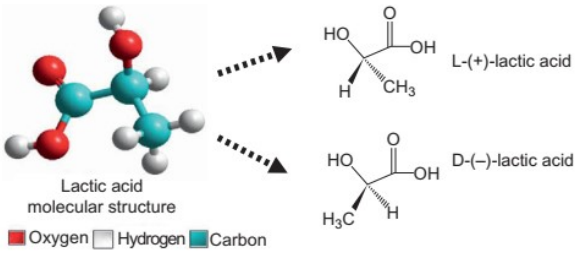


Figure 2. 3 Lactic acid chemical formula and enantiomers [6]

For this reason, PLA can be present as 2 optically active stereoisomers, D or L, or a racemic optically inactive mixture of the 2, DL [6]. Poly (L-lactic acid) (PLLA) and Poly (D-lactic acid) (PLDA) are semi crystalline, while poly (DL-lactic acid) is amorphous [6]. However, its structure can be modified polymerizing a mixture of L and D isomers, or also through copolymerization with other monomers, like glycolide and caprolactone [6].

PLA is generally produced in 2 ways: direct polycondensation (DP) of its monomer, lactic acid, or the ring-opening polymerization (ROP) of lactide, cyclic dimer of lactic acid, [6] as it is shown in figure 2.4.

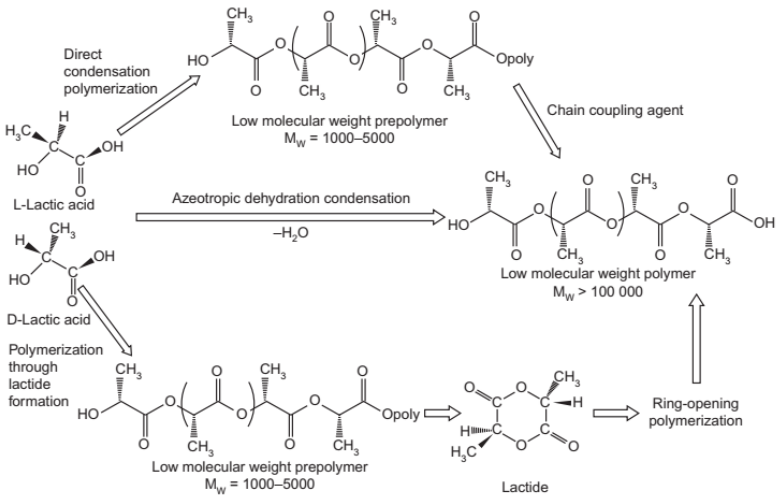


Figure 2. 4 Reactions pathways of PLA [6]

Concerning the first, lactic acid is generally produced from sugar through fermentation: there are more than 20 genera in the phylum Firmicutes forming this molecule in their metabolic process [6]. Among those, the most significant is *Lactobacillus* genera, anaerobic bacteria that survive at 5-45°C and mildly acidic conditions (pH 5.5-6.5) [6]. Their metabolic substrate includes different types of carbohydrate-rich materials, leading to lactic acid with various yields [6]. Once lactic acid is formed and recovered, the direct

condensation polymerization takes place, resulting in a low molecular weight polymer with weak and brittle properties [6]. This is the reason why, industrially, this way is not followed.

If PLA is produced via ring-opening polymerization, chain coupling agent's usage can be avoided, since the polymer has a molecular weight higher than 100000 g/mol [6]. This result is probably due to the usage of an initiator, cationic or anionic one, which forms free radicals and propagates the growth of the chain [6]. To produce lactide, a process described in the US Patent 5 274 073 called 'Continuous Process For Manufacture of A Purified Lactide' is followed. The procedure starts with lactic acid and includes a pre-treatment to remove water and to begin the polymerisation, followed by a catalytic depolymerisation and intramolecular esterification to produce lactide and the final ring opening polymerization using anionic or cationic initiators [6].

PLA is one of the most applied and used bioplastic nowadays; the categories are grouped generally as domestic, pharmaceutical/biomedical and engineering [6]. Domestic application involves apparel, bottles, cups, food packaging, etc [6]. Regarding the latter, PLA has many advantages especially for containing:

- Cheese, due to a high carbon dioxide transmittance rate, which causes less expansion of the package[7]
- Chilled or frozen products, especially dairy products, thanks to low gas and water vapour barriers [7]
- Non-carbonized beverages [7]

PLA is used in engineering and agricultural applications since it possesses optimal mechanical properties, especially a high rigidity; it is involved in automotive, building materials, electrical and electronics [6]. And finally, as a biomedical application, PLA is used in scaffolds to provide temporary structural support for the attachment and growth of tissues in surgery, as well as drug carrier [6].

2.3 PHAs

Polyhydroxyalkanoates (PHAs) are polyesters of β-hydroxyalkanoates, generally classified in three classes depending on the number of carbon atoms in the side chains: they can be short (less than 5 carbon atoms), medium (6 to 14 carbon atoms) or long chain [8,9]. In figure 2.5 is represented the simplest version of them, Polydroxybutyrate (PHB), an isotactic polymer with mechanical properties like the ones of polypropylene and polystyrene, but a tendency in becoming brittle at room temperature conditions storage [9].

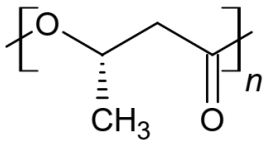
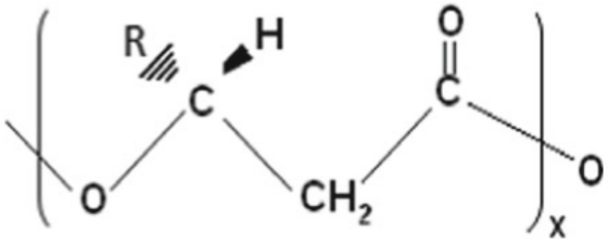


Figure 2. 5 PHAs chemical structure, in the form of PHB

PHAs are referred as bioplastics since they are produced from bacteria in stress or growth conditions, depending on the species, using renewable resources, like vegetable oils, sugars, molasses, and waste [9]. Their characteristics and properties vary depending on the length and functional groups in the lateral chains: there are more than 150 different monomers [9],

as it can be seen from figure 2.6. The high variety in composition depends on the substrate used, on the fermentation conditions (pH, temperature, etc.) and modes (batch, fed-batch, continuous) and on the microorganism responsible [9].



X varies from 100 to 30,000

R = hydrogen	Poly (3-hydroxypropionate);	R = methyl	Poly (3-hydroxybutyrate)
R = ethyl	Poly (3-hydroxyvalerate);	R = propyl	Poly (3-hydroxyhexanoate)
R = pentyl	Poly (3-hydroxyoctanoate)		

Figure 2. 6 PHA chemical structure [9]

Each specie has its own metabolic pathway to produce PHAs; anyway, the most well-known ones are three [9]:

1. Starting from sugar, producing acetyl coenzyme A (acetyl-Co-A) and then PHAs [9]
2. Starting from fatty acids, through a fatty acid β oxidation, producing (*R*)-3-Hydroxyacyl-Co-A [9]. From this PHA synthases (PhaC) is produced, which leads to PHAs in the end [9].
3. Starting from sugars but producing fatty acids from these and then following pathway 2 [9].

Despite the high number of microorganisms, that can produce PHAs as part of their metabolism, the industrial production remains low and expensive. This can be explained by three factors: type of substrate, low yield and low production rate and difficult extraction and recovery processes [9]. The cost of substrate contributes to 40-50% of the total PHAs production expense [9], but it can be reduced if inexpensive carbon substrates, such as molasses, cheese whey, waste vegetable oils, crude glycerol, agricultural wastes, palm oil or wastewater are used [9]. However, producing PHAs from waste may result in different yields and chemical characteristics, depending on the waste impurities [9]. This last problem can be overcome if laboratory tests are run before, in order to optimize the inter process, leading, in the same way, to an increase in the production itself, in terms of fermentation processes, polymer synthesis rate, efficiency of cultivation strategies [8]. Also, recombinant strains are under investigation to increase both the quantity and the production rate of PHAs [9]. Finally, new methods for the downstream recovery, using recyclable non-toxic solvents and low-cost recovery strategies, are in current investigation [9]. All these possible solutions are presented in figure 2.7.

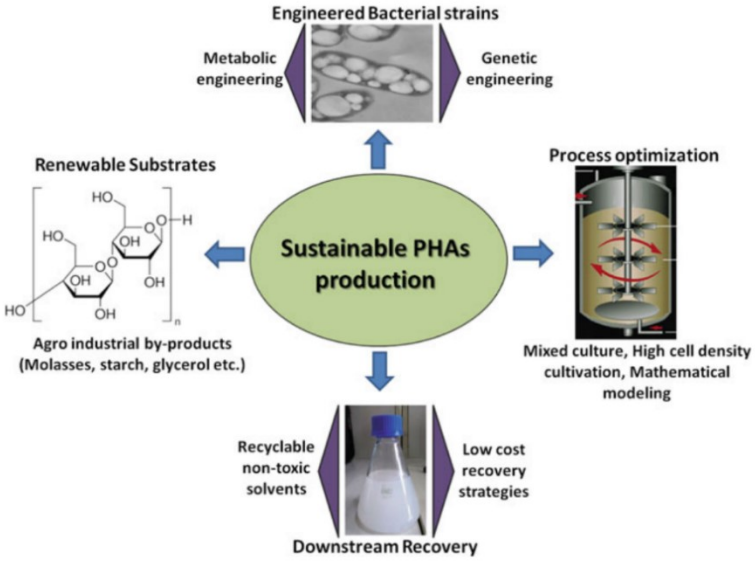


Figure 2. 7 Sustainable PHAs production solutions [9]

PHAs have mechanical and physical properties like petroleum-based polymers; however, their direct usage has been proven to be uneconomical; they are now more used as intermediate products, especially in medical sector, as antimicrobials, antibiotics, and anticancer drugs [10].

On the other hand, PHAs are used for traditional applications, such as food packaging, since they can protect from dust, contaminants, dehydration, they guarantee to retain food grade quality [10]. They can also be used to produce biofuels through the methyl esterification technique [10].

Finally, PHAs are involved, especially as copolymers, in agriculture, in mulching, nets and bags [10].

2.4 PGA

Polyglycolic acid (PGA), in figure 2.8, is the polymer of glycolic acid (GA), the simplest α-hydroxy acid, with alcohol and carboxyl groups [11]. Its chemical structure is, thus, very similar to PLA, defecting of methyl group.

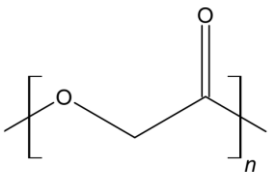


Figure 2. 8 PGA Chemical structure

The similarity among PLA and PGA are visible in their industrial production processes. The polymer is either formed by direct polycondensation reaction (DP) of glycolic acid or by ring opening polymerization (ROP) of its cyclic dimer, glycolide [11]. Other routes are also

possible, like solid-state polycondensation (SSP) of halogen acetates or formaldehyde reaction with carbon monoxide [12].

The main issue, both in polycondensation and ROP, is the production of glycolic acid, which has low yield and high costs [12]. Its production is based on petroleum sources: hydrolysis of chloroacetic acid or carbonylation of formaldehyde [12], that are the first and the third routes in figure 2.11. However now a shift towards bio-based raw materials is taking place, mainly using sugarcane, sugar beet or pineapple fermentation [11]. In all approaches, methanol is generally formed, starting either from syngas or coal or from biomass [12].

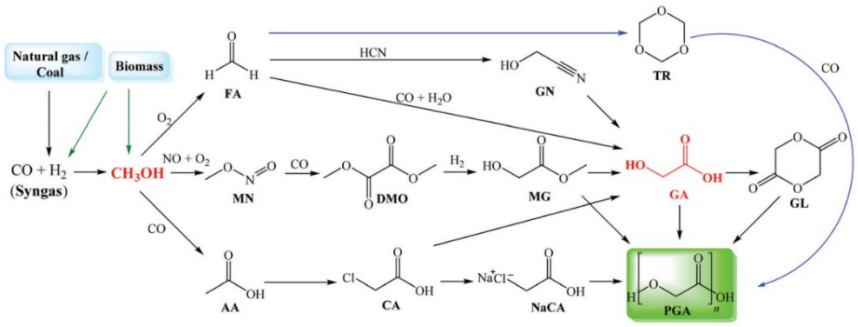


Figure 2. 9 Synthetic routes to produce PGA, from [12] (FA=formaldehyde, GN=glycolonitrile, TR=trioxane, MN=methyl nitrile, DMO= dimethyl oxalate, MG= ethyl glycolate, GA=glycolic acid, GL=glycolide, AA=acetic acid, CA=chloroacetic acid, NaCA=sodium chloroacetate, PGA=polyglycolic acid

Once GA is produced, one of the two main mechanisms, DP or ROP, takes place.

Polycondensation is not industrially followed since it produces low molecular weight PGA for two reasons:

1. Water is formed during the process, reducing the final molecular weight of the polymer [11].
2. The reaction temperature is close to the melting point, which can degrade the polymer before the process is over [11].

Ring opening polymerization is, thus, the preferable pathway followed in industry, even if high energy costs to transform glycolic acid to glycolide must be considered [12]. This process involves high pressures and temperatures and a catalyst, with a further distillation and purification treatment [12].

Once produced, PGA is employed in clinical applications, such as tissue engineering and drug delivery because of its high biodegrability and biocompatibility, [12]. Moreover, due to the high mechanical properties and gas-barrier performance, PGA is used in coatings, membranes, and packaging [12].

2.5 Bio-PET

Bio-Polyethylene terephthalate (Bio-PET) has the same chemical structure, shown in figure 2.10, as petroleum-derived PET, the difference is the source of its monomers, ethylene glycol (EG) and terephthalic acid (TA), being biomass and not fossil.

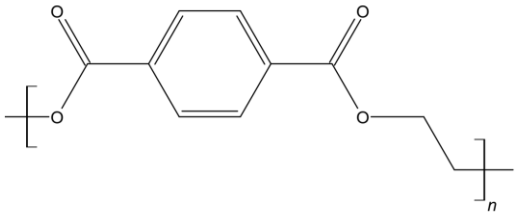


Figure 2. 10 PET Chemical structure

Bio-Ethylene glycol (Bio-EG) production is well established and is based on glucose fermentation, followed by dehydration of ethanol and oxidation of ethylene [5], as it can be seen in figure 2.11. Other possibilities, more recent and still in ongoing investigations, could be of either using sorbitol, produced via hydrogenolysis, or via metabolism of some bacteria, using xylose as substrate [5].

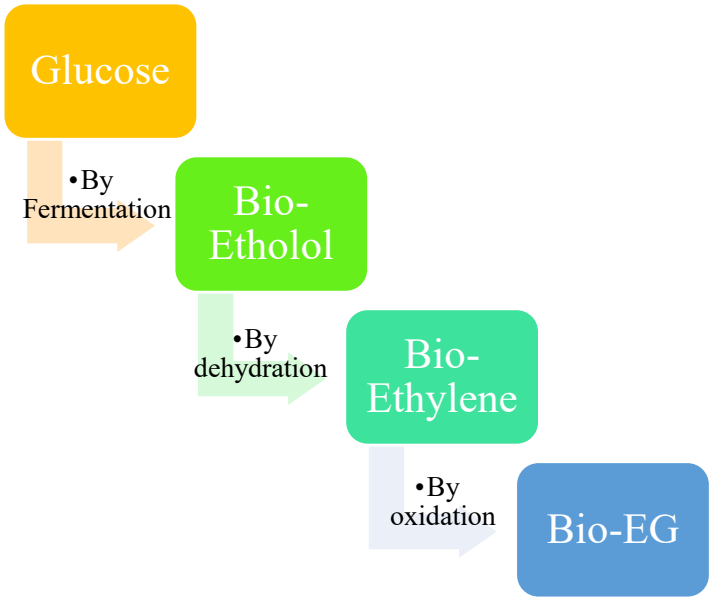


Figure 2. 11 Bio-EG production process [modified from 5]

Bio-Terephthalic Acid (Bio-PTA) is more complicated to produce completely from renewable materials; different methods are currently in research, based on iso-butanol, muconic acid, limonene, furfural, or other compounds, all shown in figure 2.12 [5].

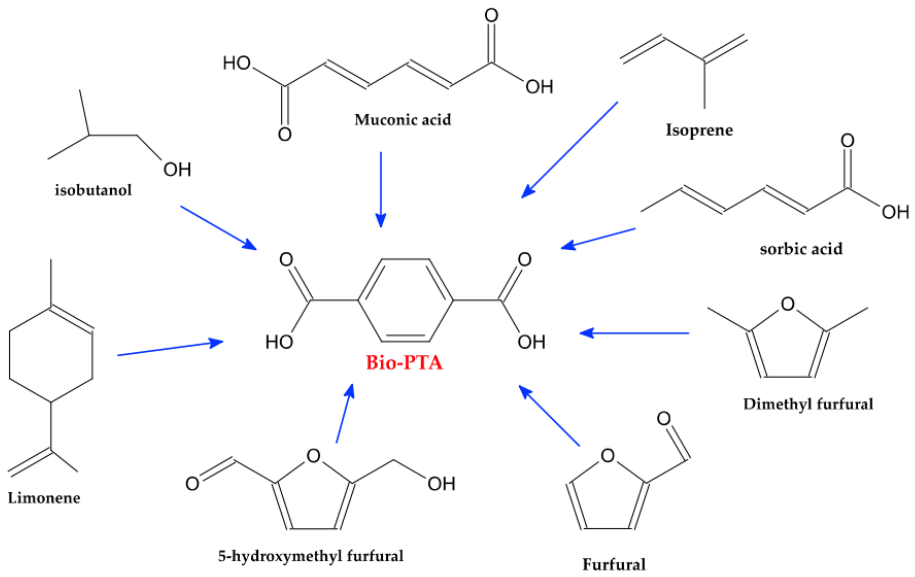


Figure 2. 12 Methods used to achieve bio-PTA, from [5]

As its petroleum-derived polymer, bio-PET possesses excellent mechanical, electrical and thermal properties [13]. PET resin is mainly used for bottles purposes and recent Life Cycle Assessment (LCA) studies confirmed that those bottles have 12% less global warming impact than petroleum PET based ones, however the results depend still on the biomass feedstock availability [14].

3. State of the art on degradation of plastics

Bioplastics, as well as traditional plastics, must be processed after their usage; as already mentioned, this treatment could be challenging, since it depends on the structure of the material and on its contaminants. Apart from traditional landfilling or incineration, which are not proper recycling methods, other techniques have been used and evaluated, as it can be seen from the figure 3.1, which shows an envisioned plastic value-chain system that could be achieved in 2030. Here it is clear that thermal, chemical (with solvolysis and dissolution) and mechanical methods must be enhanced, to depolymerize the polymer into its constituents, that can be reused.

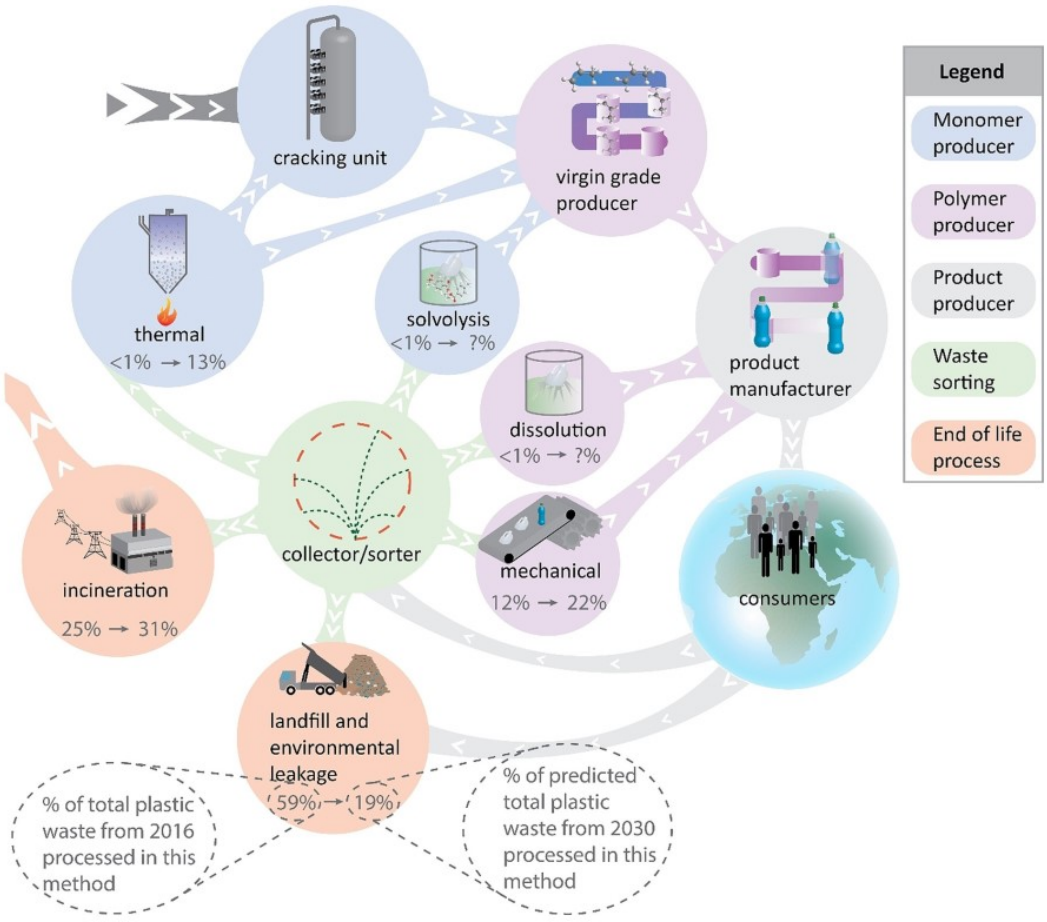


Figure 3. 1 Envisioned plastic value-chain to enhance the transition to circular economy [2]

In the following paragraphs a short evaluation of traditional and innovative methods will be provided, as well as a short overview of the most used methods for the bioplastics described in the last chapter.

3.1 Thermal methods

Thermal treatments, and especially pyrolysis, involve high temperature and absence of oxygen in a nitrogen atmosphere, producing organic vapours, char and gases, which can be then condensed to an oil with properties like crude oil , from which constituent monomers can be produced again [2].

To reduce the process temperatures and improve the selectivity of the process, catalysts can be used in the so-called catalytic pyrolysis [2]. The issue in this case is the high viscosity, the low thermal conductivity and the long molecular chains that could decrease the contact area between the catalyst and the polymer chain and inhibit the heat and mass transfer [2]. Although some new catalysts design has been produced, like nanoparticles or nanocrystalline materials, the search for a more economical, selective, stable, and active catalyst is still ongoing [2].

Thermal and catalytic pyrolysis proceed differently: the first follows a free-radical mechanism, whereas the second proceeds through a combination of free-radical and carbocation mechanism [2].

Another possible way of having pyrolysis is to substitute nitrogen with hydrogen as an inert gas, the so-called hydrocracking [2]. In this way coke formation is reduced, as well as reaction temperatures, all leading to a higher lifetime of the catalyst [2]. The final product will be constituted more of alkanes than alkenes, meaning that hydrogen saturates the double bonds present [2].

Nowadays pyrolysis process is not well established, even though there are a few plants, mainly pilot ones, that are using this mechanism. For example, the Dutch/Indian company Patpert Teknow Systems has 40 installations applying the catalytic process: the capacity varies between 110 and 7300 tons/year [2]. Their system works with a silica/alumina catalyst, at a temperature of 350-360 °C; the resulting wax fractions are entering a second reactor and then a fractionation column with a catalyst fixed bed [2].

3.2 Chemical methods

Chemical methods include various forms of processes, all involving chemicals, to depolymerize the starting product. Some of these mechanisms are already established and there are plants operating with them, like solvolysis, dissolution and precipitation and upcycling, others are more innovative, and their development is still ongoing, such as mechanochemistry, ambient-temperature photo-reforming and biotechnology applications. In the next paragraphs an overview of all those processes, old and new, will be presented, as well as a model of PLA hydrolysis, in order to give an example on how it is possible to operate with kinetics constants to design this process, which is applicable also for other bioplastics.

3.2.1 Solvolysis

Solvolysis is a general term used to express the complete or partial depolymerisation of polymers with or without a catalyst [1]. In this wide range we can find hydrolysis, glycolysis or alcoholysis, but also phosphorolysis, ammonolysis, aminolysis [2].

Hydrolysis is the depolymerization via water molecules; thus, it includes two steps: diffusion of water molecules inside the matrix and reaction; these two are generally occurring simultaneously, but, if the first is too slow, the reaction will mainly occur in the surface, otherwise, it will happen in the bulk [1].

Glycolysis is applied in the chemical recycling of polyesters and it consists in the insertion of a glycol into the polymeric chains [1].

Alcoholysis involves alcohol as a nucleophile, mainly used in the polyester's transesterification [1].

Less used, but sometimes followed, are phosphorolysis, ammonolysis and aminolysis, having the depolymerisation using inorganic phosphate, ammonia or amines.

The advantage of solvolysis is that resulting oligomers and monomers may be used for further applications after a purification step [2]. Although the procedure seems promising, there are some issues regarding (1) how to separate the liquid cleavage agent and the by-products, (2) the small contact area between the liquid cleavage agent and the solid polymer and (3) the recovery of dissolved catalyst [2]. Some resolutions have been proposed:

1. The separation could be obtained by crystallization, distillation or by liquid extraction with water [2].
2. One option could be of using reactive extrusion or a phase-transfer catalyst or performing the reaction in supercritical fluids [2].
3. Catalyst recovery can be solved by using solid-acid catalysts and manganese oxide-based catalysts [2].

3.2.2 PLA Hydrolysis

To further understand solvolysis principles, a kinetic model of PLA hydrolysis will be shown. Polylactic acid (PLA) is a highly hydrolysable polymer because of its ester bonds. Its hydrolysis proceeds either at the surface or within the bulk, mainly because of the rate of water diffusion, but also because of its chemical composition and its crystallinity, its molecular weight and molecular weight distribution, its chain orientation, the presence of residual monomers and the environmental degradation conditions, like temperature and pH [15].

As it was said in the last paragraph, hydrolysis is a process occurring in stages [15]:

1. Diffusion of water in the bulk
2. Hydrolysis of the chains in the amorphous region
3. Formation of oligomers
4. Hydrolysis in the crystalline phase, thanks to an autocatalytic mechanism due to the presence of acidic products and carboxylic acids

The higher the temperature the faster the degradation, as for almost every reaction, following the Arrhenius-like dependence of the kinetic constant [15]. The pH vs kinetic constant behaviour shows that there is a minimum in the activity at pH 4.5: this happens since the parameters affect both the reaction mechanism, since hydrolysis is catalysed by acids and basis, and the reaction kinetics [15].

Considering the characteristics of the polymer, molecular weight influence is important below $8*10^4$ g/mol: there, the degradation accelerates, due to increased molecular mobility, higher density of hydroxyl groups and hydrophilic terminal carboxyl and formation of water-soluble oligomers and monomers [15].

Another parameter that must be considered is the crystallinity. As it was said in the description of hydrolysis steps, the reaction begins in the amorphous regions and not in the crystalline ones, since there are difficulties for water molecules in entering these rigid structures; this leads to an increase in the degree of the crystallinity as far as the reaction proceeds in the other parts [15]. Then, the hydrophilic terminal groups of the crystalline regions are included in the next amorphous area, increasing even more the degradation rate due to diffusion of water molecules into the bulk material [15]. Furthermore, these terminal groups are places where lactoyl-acid and lactic acid are formed: they catalyse the hydrolytic degradation of PLA [15].

The crystallization evolution during hydrolytic degradation can be explained with the following method proposed by Gorrasi and Pantani [15]. X_c is the degree of crystallization:

$$X_c = \frac{M_c}{M_t} = \frac{M_c}{M_c + M_a} \tag{3.1}$$

Where M_t is the mass of the sample, M_a, and M_c the masses of the amorphous and crystalline regions.

If the mass of the crystalline region is constant, because the reaction is only taking place in the amorphous regions, making the time derivate of the equation 3.1 gives:

$$\frac{dln(X_c)}{dt} = \frac{dln\,(\frac{M_o}{M_t})}{dt} \tag{3.2}$$

Where M_0 is the initial mass of the sample. So, if the mass of the crystalline parts inside the sample does not change, then plotting the 2 terms of the equation 3.2 will give a linear relationship with slope equal to 1; a larger slope will mean an increase of crystallinity, a lower one will mean an erosion of crystalline parts. The results obtained by experiments performed by Gorrasi and Pantani [15] showed that at the beginning the slope was higher than 1 and then it became equal to 1, thus indicating that crystallinity increased for the erosion of the amorphous parts. Then it became lower than 1, meaning that also the crystalline regions were attacked by hydrolysis.

As it was explained in the last chapter, PLA monomer, lactic acid, is an enantiomer: the quantity of L-Lactide is affecting the crystallization rate and, thus, the degradation itself, as it was explained before. From the measures [15] it seems that PLA containing both enantiomers is the weakest one, because of lower intermolecular bindings, followed by a blend of the single ones (higher forces between the chains in the blend).

Kinetics of hydrolytic degradation

Gorrasi and Pantani [15] developed a model describing the kinetics of PLA hydrolysis to understand where the hydrolysis can take place. The simple reaction taking place is:

$$H_2O + ester \leftrightarrow -COOH + -OH \tag{3.3}$$

The concentration of carboxylic end-groups, C_C and the esters C_E is:

$$C_c = \frac{\rho}{M_n} \tag{3.4}$$

$$C_E = \frac{\rho}{M_n}(DP - 1) \tag{3.5}$$

Where, ρ is the density of the polymer, M_n is the change in number-average molecular weight at a reference temperature T_{ref}, DP is the average degree of polymerization (equal to M_n/M where M is the molecular weight of the repeating unit).

Experimental results [15] showed that the degradation was autocatalyzed, since the produced oligomers had a carboxylic acid terminal, which increased the acidic part. Thus, the reaction rate depends not only on the reagents of reaction 3.3, but also on the dissociated carboxylic end groups. This is shown in equation 3.6, where the acid dissociation constant K_a is proportional to C_H, concentration of positive ions, acids, and C_{-COO-}, concentration of the dissociated carboxylic acids.

$$K_a = \frac{C_H + C_{-COO-}}{C_C} \tag{3.6}$$

Providing a mass balance over the polymeric chains:

$$\frac{dR_S}{dt} = K_h C_E C_A C_C^a + K_t C_E \tag{3.7}$$

Where the first member is the rate of scission of polymer chains, C_A is the water concentration, a is a constant describing the possible autocatalytic effect (generally more important at the end of the reaction), K_h is the hydrolysis constant and K_t takes into account the thermal degradation.

If only the carboxylic groups of the oligomers are assumed to be catalysts, then also a rate of production of those must be considered. It was empirically observed that there is a correlation between oligomers production rate R_{ol} and the scission of polymeric chains R_s:

$$\frac{R_{ol}}{C_{E0}} = \alpha(\frac{R_s}{C_{E0}})^\beta \tag{3.8}$$

Where C_{EO} is the initial concentration of ester bonds and α and β are two empirical constants.

If, then, a balance over the oligomers is done, the following equation is obtained:

$$\frac{\delta C_{ol}}{\delta t} = \frac{dR_{ol}}{dt} + \overline{\nabla} \cdot D_{ol} \overline{\nabla} C_{ol} \tag{3.9}$$

Where D_{ol} is the effective diffusivity of the oligomers inside the sample, generally depending on the amount of degradation and of crystallinity degree.

At the same level, also the water diffusion can be evaluated:

$$\frac{\delta C_A}{\delta t} = -K_h C_E C_A C_C^a + \overline{\nabla} \cdot D_A \overline{\nabla} C_A \tag{3.10}$$

Where D_A is the water diffusivity inside the sample.

Hydrolysis takes place in two parts: in the bulk and in the surface of the sample. The choice is depending on water diffusion and polymerization degradation itself: if the first is faster, then the hydrolysis happen mainly in the bulk, otherwise the erosion moves towards the interior [15]. This mechanism is then intrinsically related to water mass balance, 3.10.

Based from experimental data, another model was obtained by Gorrasi and Pantani [15]. The first term of 3.10, expressing the reaction rate will be:

$$\rho \frac{d}{dt}(\frac{1}{M_n}) e^{-E_R(\frac{1}{T} - \frac{1}{T_{ref}})} \tag{3.11}$$

While the diffusion, the second term of 3.10, can be estimated as:

$$\frac{D_{A,ref} C^*_{A,ref} e^{-(E_D + E_C)(\frac{1}{T} - \frac{1}{T_{ref}})}}{\delta^2} \tag{3.12}$$

Where $C_{A,\ ref}^{*}$ is the equilibrium water content at the reference temperature, $D_{A,ref}$ is the diffusivity of the water at the temperature of reference, and E_D and E_C are constants describing temperature effect on diffusion and sorption.

To evaluate the distance from the surface at which the diffusion and the reaction are the same it is enough to equalise equation 3.11 and 3.12, obtaining

$$\delta \approx \sqrt{\frac{D_{A,ref} C_{A,ref}^{*} e^{-(E_D+E_C-E_R)(\frac{1}{T}-\frac{1}{T_{ref}})}}{\rho \frac{d}{dt}(\frac{1}{M_n})}} \tag{3.13}$$

Experimental results showed that at 55-60°C, this distance was 5 mm [15].

This model, although simplified, shows that the place where the hydrolysis takes place depends on many parameters, such as temperature, diffusion, water concentration, density, molecular weight.

3.2.3 Dissolution/precipitation processes

Dissolution processes followed by precipitation are generally involved in the separation of different polymers applied in a blend, in the removal of colorants and other additives [2], like for examples in milk or juice containers. To do so, a single solvent or a mixture of a solvent and an antisolvent may be used: the latter combination is working on a dissolution followed by a recovery process [2].

This procedure is, however, time and energy consuming, and it is difficult to remove completely the solvents [2]. These issues could be overcome if a supercritical solvent, such as dimethylether is used: it will be easily separated by evaporation when the pressure decreases [2].

Even though some plants are already operating with this technique, such as the NewCycling process developed by APK AG, separating multilayer films of aluminium and Polyethylene and Polypropylene [2], new companies are willing to use it and increase even more the research. This will mainly focus on the solvent choice, which must be selective towards the polymer, not hazardous and easily evaporable to be recovered at the end [2].

3.2.4 Upcycling

Upcycling is the process by which plastics are transformed into value-added products, such as solvents or coatings [2]. This is generally done similarly to solvolysis; the difference is, however, that in this case the depolymerization is not complete: the final products are not monomers, but oligomers [2]. These latter can be then used in two ways [2]:

1. In the combination of partly depolymerized polymers and renewable sources, like in the synthesis of fibre-reinforced plastics starting from depolymerized PET and bio-derived olefin acids [2].
2. In the post-polymerization modification [2].

3.2.5 Mechanochemistry

Mechanochemical treatment is a new efficient method that applies mechanical stresses to cleave the polymeric chain, which can be used to form cross-linking and cross-polymerization or to depolymerize the plastic [2]. It can be also used to pre-treat waste plastics before other treatments, especially when it comes to pyrolysis [2].

This technique is simply done using shear-reactors, ball mills or sonication; one issue is that there is a limit in the molecular weight, under which it is not possible to cleave the chain mechanically [2].

3.2.6 Ambient-Temperature Photo-Reforming

Photo-reforming is an innovative method that uses the direct photonic energy in ambient-temperature conditions with inexpensive catalysts and allows the reforming of polymers [2]. It has been reported that Ni_2P supported on cynamide C_3N_4 catalysed PET and PLA cut with enough stability [2]. In this way, more expensive catalysts, generally used in chemical recycling, like Cd-based photocatalysts can be avoided [2].

3.3 Biotechnological methods

Biotechnology method is based on the depolymerisation of plastics, done by microorganisms and their metabolism, able to do it in order to use the substrates to grow. This possibility relies on their enzymes, natural catalysts able to speed up the depolymerisation. Nowadays this procedure is still under ongoing research, but, once proven, it will be useful in the management of end-of-life plastics treatment, since it is not hazardous, and it does not involve toxic compounds or hard operative conditions. Therefore, this research, with the purpose of showing the degradability of some new bioplastics, will focus on this technique.

Enzymes in cells and microorganisms have been shown to be able to degrade plastic waste, especially polyesters, since they have a familiarity with natural ester bonds that microorganisms use as carbon and energy source [16]. To show the mechanism behind this process, PLA biodegradation will be described, as it has been done with hydrolysis.

3.3.1 Biodegradation of PLA and affecting properties

Biodegradation of PLA is a process by which microbial enzymes are catalysing the hydrolysis of its ester bonds; this is generally done in nature, but the same principle is followed also in biotechnology recycling technique. The technique can be done in two ways: by using direct enzymes, as it will be done in this research, or by growing some polyesters degrading microorganisms.

In both cases the used enzymes are generally exo-enzyme present in microorganism, natural or recombinant, that uses monomers, coming from hydroxylation, to produce energy and inorganic molecules [2].

Although the process is complicated, it may be seen as an heterogenous reaction: the substrate, in this case the polymers, must reach the catalysts, the enzymes, where the reaction takes place. Thus, following the mathematical model proposed by Gorrasi [15], a mass balance over mass metabolized will be:

$$r_m S = -\rho S \frac{d\delta}{dt} + j_M S \tag{3.14}$$

Where j_M represents the diffusion of the polymers from the sample towards the enzymes, r_m is the reaction term, expressed per mass of polymer metabolized per unit time and surface, while the second term represents the erosion, with δ thickness of the sample and S the sample surface.

It is generally assumed that the reaction rate is constant and inversely proportional to δ, while the diffusion is evaluated to be:

$$j_M \approx \frac{D_{ol}}{\delta} C_{ol} \tag{3.15}$$

Where C_{ol} is the concentration of oligomers in the sample.

Even though from equation 3.14 is not possible to see it, biodegradation, as well as hydrolysis, depends on many factors, mainly divided in: [16]

1. Exposure conditions
    a. Abiotic (temperature, pH, and moisture in the compost as well as UV)
    b. Biotic (extracellular enzymes, hydrophobicity, and bio surfactants)
2. Polymer characteristics (flexibility, crystallinity, morphology, functional groups, crosslinking, molecular weight, copolymers, blend, tacticity, and additives)

The parameter that affects both erosion and diffusion terms in equation 3.14 is, however, still crystallinity. In both cases, the higher it is, the lower the term will be.

- For the reaction rate because crystallinity makes the microorganisms less prone to reaction [15]
- For the diffusion term because hydrolysis is slower in crystalline regions and the diffusion parameter itself is lower for semi crystalline polymers [15]

This is true for every polymer, no matter if it is biobased or coming from petroleum sources: the chemical structure is just one of the many parameters that can change the reaction rate, especially when enzymes and microorganisms are applied.

3.4 Optimal degradation methods for bioplastics

The currently used bioplastics presented in chapter 2 are all biodegradable. Nevertheless, their recycling treatment is not always exploiting this ability, mainly for time issues. Despite that, new solutions have been applied, using the innovative methods described above.

3.4.1 PLA Degradation

PLA circular recycling is mainly based on solvolysis, especially hydrolysis and alcoholysis, with the main difference between those being the presence of catalysts, since the first is an autocatalyzed process, while the second needs an external catalyst, but its results have a higher chemical value [1]. In both cases, the product monomers can be converted again in lactide, the starting material used in PLA industrial production processes [1].

Biodegradation of PLA, although possible, as it was described in the previous paragraph, is difficult due to the low quantity of microorganisms able to cleave its bonds [1].

3.4.2 PHA Degradation

A different approach is the one generally followed for PHAs [1]. Here, mechanical, and chemical recycling routes are not followed because they are expensive [1]. The choice is between pyrolysis, in case some value-added materials are wanted, and biodegradation [1]. This last method is more and more chosen since there are many microorganisms that use PHAs as substrate to grow [1].

3.4.3 PGA Degradation

PGA is mainly used as a copolymer with *L*-lactide and in biomedical applications [1]. Thus, its recycling is not necessary since it biodegrades inside the body. For this reason, biodegradation and biocompatibility must be assured: this is depending on the ratio between PGA and *L*-lactide, with PGA having the highest absorption and *L*-lactide the lowest one [1].

3.4.4 Bio-PET Degradation

Bio-PET optimal recycling route is based on a possible mechanical recycling, as for petroleum-based PET, for as long as possible; then, once its properties become downgraded,

the chemical method is followed [1]. Hydrolysis and alcoholysis are not the first choice, due to harsh operative conditions applied and the use of catalysts in the second [1].

The most widely used method is, thus, glycolysis, since it has the lowest activation energy and the purification step is easily producing just Bis (2-Hydroxyethyl) terephthalate (BHET), from which it is possible to have again PET [1].

However, it is also interesting to underline that biodegradation, especially using direct applications of enzymes, is gaining importance after the discovery of a microorganism that uses PET as primary substrate. This will be explained in chapter 4.

Table 3. 1 Different bioplastic normal degradation procedures

Bioplastic	Mechanical Treatment	Chemical Treatment	Thermal Treatment	Biological Treatment
PHA	No, high cost	No, high cost	Pyrolysis	Easily degradable
PLA	Yes, but downgraded	Yes, hydrolysis without catalyst or alcoholysis with catalyst		Yes, but only with specific microorganisms
PGA				Yes, but depending on the composition of the copolymer
Bio-PET	Yes	Hydrolysis and alcoholysis but high temperatures and pressures. Glycolysis most applied method		Yes, PETase

4. Enzymatic degradation

Biodegradation, especially using direct applications of enzymes, could be one innovative approach to solve the plastic recycling problem, although industrial plants are not yet using it. Thus, the experimental work of this research will focus on biodegradability tests performed on bio-polyesters that will be shortly described in this chapter, with the aim of supplying materials and results for further research.

During these tests, enzymes able to degrade ester bonds will be used: their structure as well as their reaction mechanism is here described.

4.1 PET degradable enzymes

Plastic accumulation in the environment is an issue that can cause real damages to the biosphere; however, the high quantity of polymers in the soil and water is making microbes and other microorganisms adapt in modifying their metabolism to be able to use those materials as substrate for their sustenance [17]. Thus, they produce enzymes, able to hydrolyse biopolymers bonds, especially the ones of polyesters. For this ability those enzymes are generally referred as hydrolases, the most known of those being cutinases and lipases.

One of the most abundant plastic used today is PET [17]. As already described, PET is a polyester aromatic polymer, so it is more difficult to degrade compared to other polymers, due to the steric hindrance of the benzene ring.

Until recently, PET biodegradation was not a recycling applied method since there were not many well-known microorganisms able to use it as a substrate; however, in 2016 Yoshida et al. [17] found a bacterium, *Ideonella sakaiensis* 201-F6, able to degrade it thanks to two enzymes, as it can be seen from figure 4.1. The first one, called PETase, converts PET to mono (2-hydroxyethyl) terephthalic acid (MHET), terephthalic acid (TPA) and bis (2-hydroxyethyl)-TPA (BHET) [17] . Then the second, MHETase, transforms MHET into TPA and ethylene glycol (EG), the two monomers of PET [17].

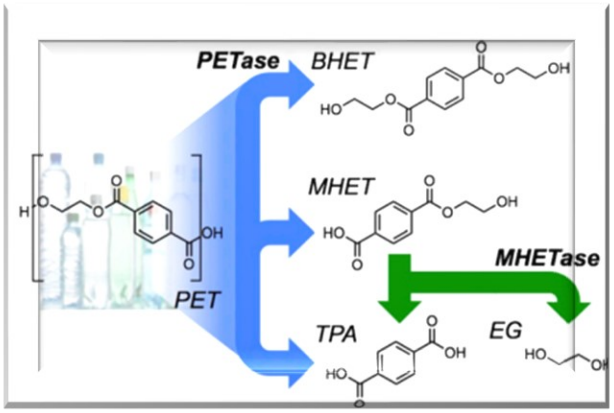


Figure 4. 1 PETase and MHETase reaction pathways [17]

This discovery gave impulse in investigating its mechanism and structure, as well as using it in practical applications. This investigation includes another enzyme type, cutinases,

which, however known for a while as catalysts in other reactions, gained interest in the last few years also on PET degradation. For these reasons, it seems appropriate to discuss both, before applying in the experimental work.

4.2 PETase

4.2.1 Chemical Structure

Numerous studies have been started since 2016, to be able to understand the chemical structure as well as the degradation mechanism applied by PETase. One of these, led by Austin et al. [17] showed through high-resolution X-ray its crystal structure. PETase is a classical α/β hydrolase enzyme, with 8 β-strands and 6 α-helices [17]. The active site, shown in the red circle in figure 4.2, is composed of a catalytic triad of Ser160, Asp206 and His237 [17]. These catalytic residues are positioned in loops, with the nucleophilic serine in the "nucleophilic elbow" in the consensus sequence (Gly-X1-Ser-X2-Gly), with X1 containing a tryptophan residue, Trp159, that gives an extended hydrophobic surface adjacent to the active site [17] . PETase has also two disulphide bonds, one near the active site and one near the C terminus of the protein [17].

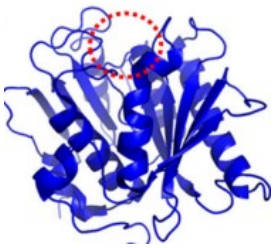


Figure 4. 2 Cartoon representation of the PETase structure at 0.92 Å resolution [17]

4.2.2 Mechanism

PET hydrolysis catalysed by PETase is still not known in all details, but some mechanisms have been proposed and they will be now summarized.

The first is the one proposed by Chen et al. [18]: they tried to explain also how the enzyme is bonding to the substrate and to the product. They analysed the molecular docking of a mutated PETase, R103G, on (1-(2-hydroxyethyl) 4-methyl terephthalate, HEMT, analogue to PET since it has an ester moiety, and *p*-nitrophenyl (pNP), terephthalic acid analogue. The enzyme was, thus, not the wild type since they wanted to be sure that the active site would not have been occupied by the next protein sequence, which sometimes happens [18].

HEMT carbonyl group was next to Ala131, thus the nucleophilic attack by Ser131 was facilitated, as it can be seen from figure 4.3 (a) [18]. In this way, the O atom of the carbonyl group was in the oxyanion hole (in the main chain NH groups of Met132 and Tyr158). Ile179 and Met132 gave hydrophobic contacts, Trp156 ring provided T-stacking for the aromatic moiety of the substrate [18].

pNP bound in the same place as HEMT, but it was rotated of 36° and shifted from the catalytic centre of 2.3 Å: the benzene ring was then π-stacked by Trp156 indole ring, as it is shown in figure 4.3 (b) [18].

The reaction mechanism, in figure 4.3 (c), would have been the following. PET bound like HEMT, then the hydrolytic reaction took place: the acyl-enzyme intermediate was formed, and water cleaved the ester bond by a second nucleophilic attack [18]. The benzoic acid

group formed then a broader planar surface and stronger π-stacking interactions with Trp156, as it was described for pNP [18]. The product was rotated and exited the active site [18].

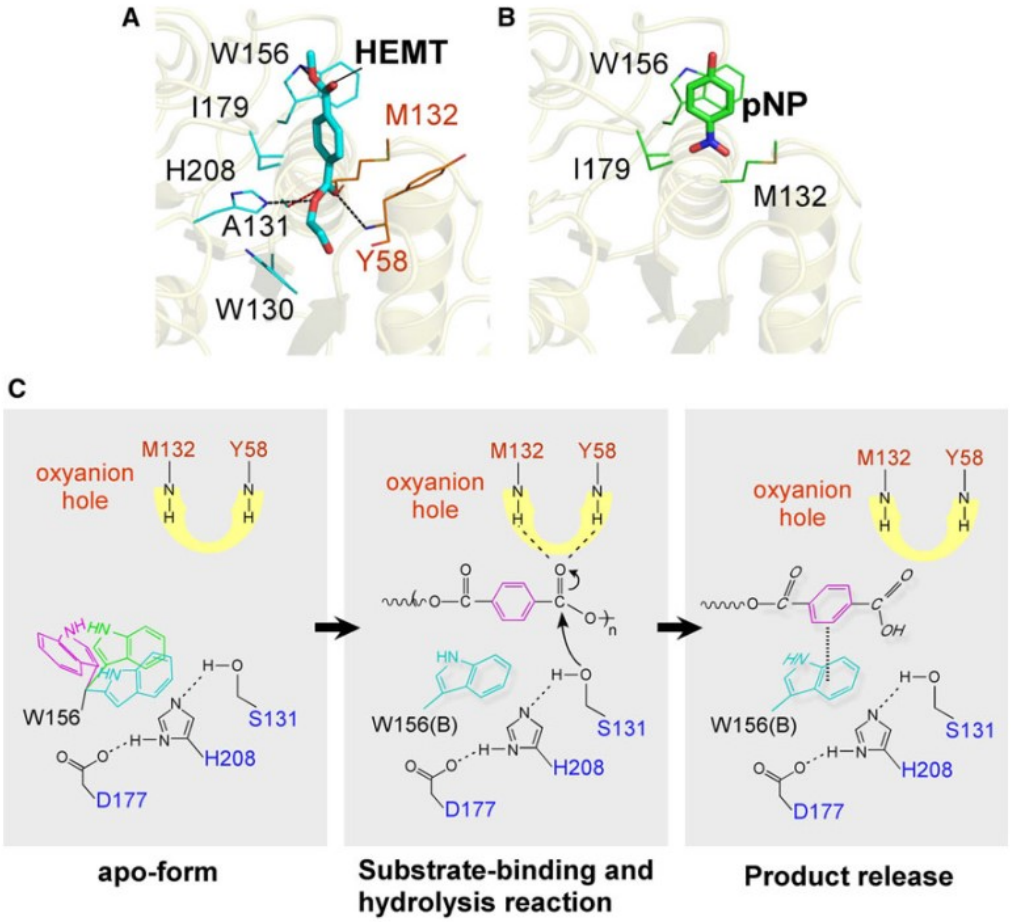


Figure 4. 3 Complex structure and catalytic reaction of PETase. (A) represents the active site interaction of PETase with HEMT and (B) with pNP. (c) mechanism of action of PETase [18]

A more specific approach, which also considers the intermediates, is the one proposed by Joo et al. [19]. They divided the binding site into two subsites (I and II): those could have bound one and three MHET moieties respectively, as it can be seen from figure 4.4.

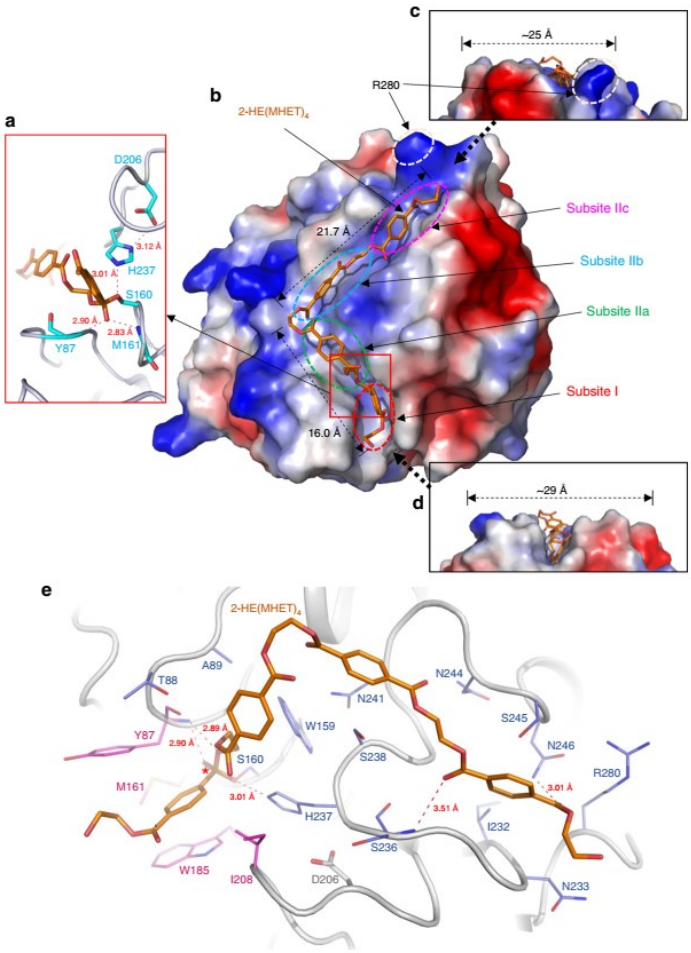


Figure 4. 4 Representation of PETase active site. (A) Catalytic triad and docking model of the reaction intermediate. (B) Substrate binding site. (C, D) Side views of the substrate binding mode of PETase. (E) Residues involved in the active site of PETase [19]

PET degradation started with PETase binding the substrate surface through flat hydrophobic contacts. Then the process was divided in two:

1. First, 4 MHET moieties were bound to each substrate binding site the scissile ester bond positioned between subsite I and II near the Ser160 residue [19]. One ester bond was hydrolased, two PET chains with different terminals were formed: TPA from subsite I and HE from subsite II [19]. The whole process is illustrated in figure 4.5 and it is known as nick generation step (a).
2. At this point, there were chains with HE as a terminal and chains with TPA terminal and they both were transformed into MHET monomers, as it is seen in figure 4.5 (b), using subsite I and II [19]. For the HE-terminal one MHET monomer and a $^{HE}PET_{n-1}$ were formed (with this last polymer probably digested in the same way after since it has the same terminal) [19]. For the TPA-terminal the products were $^{HE}PET_{n-1}$,which underwent the same process as before, and a TPA molecule [19]. Sometimes ^{HE}PET and ^{TPA}PET were digested in the reverse direction, even with lower yield than the other possibility [19]. A variety of PET monomers and dimers, like 2-HE $(MHET)_2$, $(MHET)_2$, MHET and BHET were produced: finally, they are digested to MHET, TPA and EG [19].

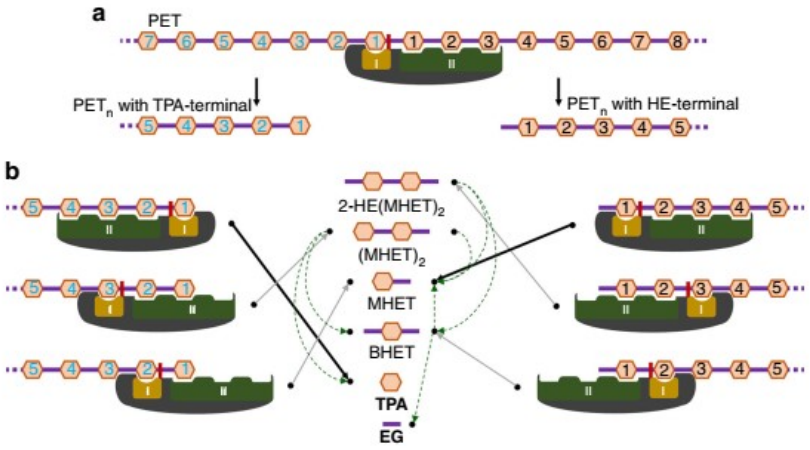


Figure 4. 5 PET degradation process. (a) Nick generation step (b) Terminal digestion step [19]

However, Wei et al. [20] investigated the results from this last research and revealed that the conformational fitting of the substrate made by Joo et al. could not have described the motions and conformations of the polymeric chain. Moreover, they pointed out that this mechanism was complicated and difficult to occur at ambient temperature, since it required numerous binding at the same moment [20]. Thus, they presented a study based on nuclear magnetic resonance (NMR) analysis of amorphous PET. They determined that the probability distribution of the OC-CO torsion angle phi was of +-70° with a ratio of 9:91 at 30°C, instead of 180° [20], as Joo reported. Therefore, they hypothesized that the binding of PET to PETase was depending more on the presence of weak interactions with the aromatic phenylene units and hydrophobic amino acids [20].

4.2.3 PETase modifications

Wild-type PETase efficiency depends widely on the operational conditions and it is selective towards precise substrates, thus, many researches were led to change some of its characteristics and make it more flexible. Austin et al. [17] tested both PETase and a cutinase from *Thermobifida fusca:* they thought that modifying PETase to a more similar cutinase structure could have increased the efficiency of PET degradation. Thus, they produced a PETase double mutant, S238F/W159H, to narrow its active site, like the cutinase model, which will be described in the next paragraph. Also, they produced another mutant, W185A, to analyse the role of Trp185 dynamic residue. Differently from the model proposed by Yoshida et al., they used a substrate with higher crystallinity (14.8% rather than 1.9%). The results showed that both the crystallinity reduction, after its first increase, and the product release were higher when the PETase double mutant was applied [17]. Considering the induced fit docking (IDF) model, these results can be explained for the presence of Phe238 and its more stable aromatic interactions with the substrate in the mutant PETase, which the wild type enzyme did not have [17]. On the other hand, W185A mutant PETase had a lower performance relative to the wild-type PETase, meaning that this residue had a critical role [17].

Another study, by Cui et al. [21] proposed a mutation, called DuraPETase, by applying an innovative computational strategy named GRAPE (Greedy Accumulated strategy for Protein Engineering), consisting in a systematic analysis of the clusters and their storage in a library. This analysis was divided in three parts.

1. They computationally predicted all the potentially stabilizing mutations along the protein sequence.
2. The candidates were inspected for biophysical flaws, like internal cavities, weak interactions.
3. All the beneficial variants were stored in a library, based on their positions, efficacies, and effects.

Following this method, they found a variant, called DuraPETase, which was more efficient than wild type PETase in degrading crystalline PET films even up to 60°C, while natural enzyme could only reach 37 °C [21]. DuraPETase had a higher thermostability, higher tolerance to organic solvents and was able to degrade PET in a more uniform way [21]. Moreover, it hydrolyzed the bonds in nanoplastics, with a diameter of 50 to 100 nm [21].

4.3 MHETase

MHETase is the second enzyme that *Ideonella sakaiensis* uses to degrade PET: it converts mono-(2-hydroxyethyl) terephthalate (MHET) to terephthalic acid (TPA) and ethylene glycol (EG). However, the origin of MHETase is still not known. At the beginning, it was thought to be part of the tannase enzyme family, Block X of the α/β hydrolase fold enzyme in ESTHER database, same as some fungal and bacterial tannases and feruloyl esterases [22]. Although they shared some properties, it was shown that MHETase was not able to hydrolyse BHET, PET, pNP, aliphatic esters or aromatic ester compounds, ethyl gallate and ethyl ferulate, generally converted by those enzymes [22].

Palm et al. [22] found out the MHETase structure by MR (Molecular Replacement) with the coordinates of *Thermobifida fusca* cutinase TfCut2. The structure was similar to the esterase one since it had a lid domain between β-strand 7 and α-helix 15 of the α/β-hydrolase fold [22]. Moreover, by X-ray fluorescence spectroscopy, there was a structural calcium-binding site; one of the five disulphide bonds was next to the catalytic triad (Ser225, His528 and Asp492), visible in figure 4.6, and the oxyanion hole comprised the amide nitrogen atoms of Gly132 and Glu226, all properties shared with other esteras [22]. Considering PETase, the two enzymes only shared the α/β hydrolase fold [22].

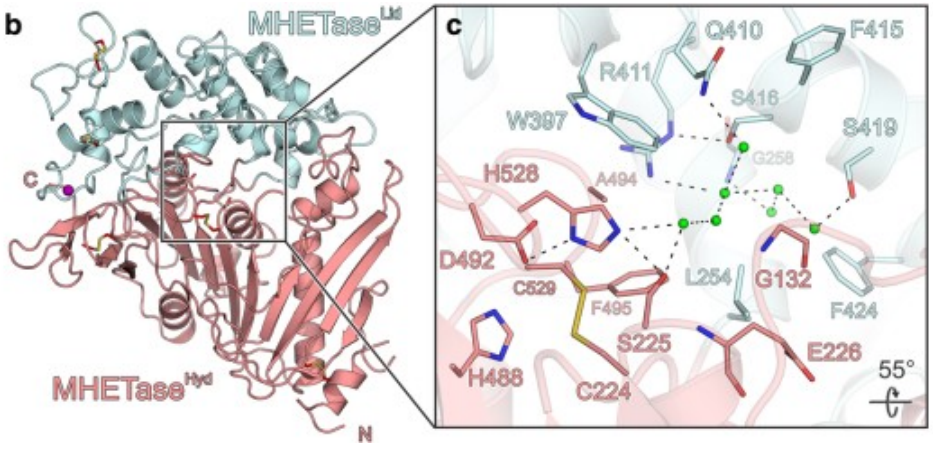


Figure 4. 6 MHETase structure (b) and close view of its catalytic triad (c) [22]

In the same paper, Palm et al. [22] investigated the substrate bound form of MHETase. The differences with the substrate free enzyme were minimal but showed that the enzyme followed an induce-fit mechanism [22]. Particularly, Phe415 rotated and opened the active

site just when the substrate was near [22]. The high selectivity of the enzyme towards MHET came from the domain lid, and not from the catalytic triad or the oxyanion hole [22].

Recent researches, led by Sagong and his coworkers [23], revealed that MHETase not only has the ability of degrading ester bonds in MHET structure, but also the terminus of PET. This function is called exo-PETase and could possibly increase even more the number of enzymes directly degrading PET [23]. However, they showed that this ability was low, but it could have been increased if MHETase would have been mutated to achieve a higher attitude in degrading PET residues with an EG terminus, rather than a TPA terminus. In other words, MHETase must achieve an activity towards BHET, a by-product of PET degradation, which has one hydroxyethyl molecules more than MHET [23].

Although the results of all the MHET involving studies seemed promising, in this research just PETase will be used as polyester hydrolysing enzyme, due to the still low understanding about this second step.

4.4 Cutinases

4.4.1 Chemical structure

In the α/β-hydrolase superfamily there are also cutinases, which name come from the plant polymer cutin, that this enzyme can hydrolase. Plant cuticle is composed of waxes and other lipids; its function is to protect the plant from dehydration and provides a barrier from pathogens [24]. The major constituent is cutin, a "hydroxylated 16- and 18-carbon fatty acids lipid linked through ester bonds" [24]. For this ability, many microorganisms use cutinases in their metabolism to disrupt this structure: they could be fungi or bacteria. Cutinases can, however, catalyse not only hydrolysis of other molecules, such as triacylglycerols and low-molecular soluble esters, but also esterification and transesterification reactions [24].

Chen et al [24] studied the structure of these enzymes and reported it. Cutinases are serine esterases belonging to the α/β-hydrolase superfamily, such as PETase [24]. Their catalytic triad is composed of Ser-His-Asp, with the first residues exposed to solvent, but, differently from lipases, they do not have a lid covering the serine; thus, they catalyse reactions involving molecules of big dimensions [24]. The structure of cutinases is dependent on their origin for what concerns the oxyanion hole, the folding, and the presence of disulphide bonds [24]. If they are fungal cutinases the residues involved in the oxyanion hole are Ser42 and Gln121, while if they are from bacteria are Met and Tyr [24]. Moreover, the structure from the bacterium *Thermobifida Alba* has a larger fold since it contains nine sheets, with two antiparallel, at the core of the protein, while fungal cutinases have just five parallel ones [24]. Finally, bacterial cutinases have a disulphide bond, which stabilizes the structure, as in PETase [24].

4.4.2 Cutinase PET degradation ability

Independently from the considered reaction and substrate, when it comes to hydrolysis catalysis, cutinases act like other α/β serine hydrolases. After the first cleavage, an intermediate linked to the serine via the carbonyl group of the hydrolasable ester is formed [24] and then hydrolased by water to be finally released. Since the research is focused on PET degradation a study regarding bacterial cutinases differences and one about fungal cutinase, when compared to alkali treatment, will be now showed.

Cutinase can have different origins and this can lead to some differences on kinetics when it comes to PET degradation: this issue was the central part of the study led by Acero et al. [25]. They based their research on three bacterial cutinases: two of them (Thc_Cut1 and Thc_Cut2) coming from the same microorganism, *Thermobifida cellulosilytica,* and the last

(Thf42_Cut1), from *Thermobifida fusca.* All three of them were very similar (94-99% homology) and they differed just for 6-18 amino acids, with the major similarity being between Thc_Cut1 and Thf42_Cut 1 (6 different amino acids) and the lowest between Thc_Cut1 and Thc_Cut2 (18 amino acids).

Their activity was measured using p-nitrophenyl acetate (PNPA) and p-nitrophenyl butyrate (PNPB) as standard substrates.

In all cases a Michaelis-Menten equation (4.1) was involved to describe their kinetic.

$$r = V_{max}\frac{[S]}{K_M + [S]} \tag{4.1}$$

Where r express the reaction rate (mol/s), $[S]$ express the substrate concentration (mol/l), V_{max} is the maximum rate (mol/s) and K_M is the Michaelis constant (mol/l), the substrate concentration at which the maximum rate is at its half. If the enzyme concentration is much less than the substrate, as it was in this case, the equation can be written as in 4.2.

$$r = k_{cat}[E]_0\frac{[S]}{K_M + [S]} \tag{4.2}$$

Where k_{cat} is the catalytic rate constant (1/s) and $[E]_0$ is the initial enzyme concentration (mol/l).

They evaluated K_M and k_{cat} with the software "Origin", version 4.10. K_M values of the three were all in a similar range (127-200 µmol/L for PNPA and 1483-2133 µmol/L for PNPB), while k_{cat} varied in two orders of magnitude (with the highest value being the one presented by Thc_Cut1 and the lowest the one of Thc_Cut2).

Performing 3PET (bis (benzoyloxyethyl) terephthalate hydrolysis, Thc_Cut1 was found to be the most efficient, with the highest content of monomers compared to the others [25]. Moreover, when the enzymes were incubated with PET films, all three of them released TPA (major product for Thc_Cut1 and Thf42_Cut1) and MHET (major product for Thc_Cut2) but not BHET [25]. These results are in accordance with the k_{cat} values: Thc_Cut1 had the highest kinetic parameter, thus, its rate went faster, and the final products contained more building block monomers than oligomers [25].

This can be explained when looking at the structure differences between the two cutinases from *T. cellulosilytica.* From the sequence alignments studies, no significant differences near the active site emerged, but the electrostatic potential and hydrophobicity on the surface were quite different and this was probably the reason of different activity [25]. The two regions with the highest content of differences were identified in figure 4.8; region 1 of Thc_Cut1 includes Ser19, Aasn29, Val30 and Glu65, while in Thc_Cut2 are Arg19, Arg29, Ala30 and Gln65. Region 2 of Thc_Cut1 comprises Ala183, Lys187, while in Thc_Cut2 are Leu183 and Arg187 [25]. It seems that Thc_Cut1 had one positive charged region and a second more hydrophobic region less than Thc_Cut2: this probably made the intermediate of the reaction more stable and the reaction itself went faster [25].

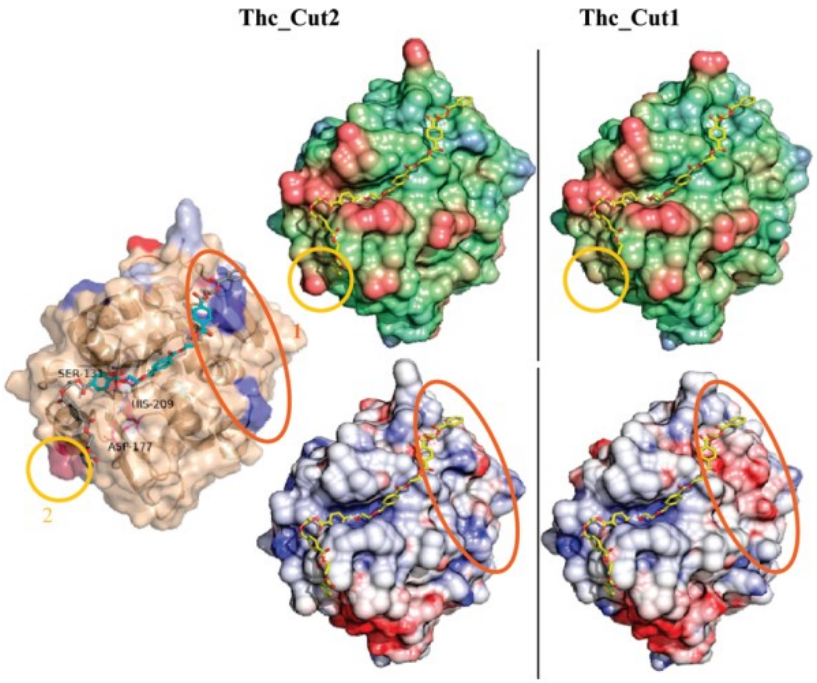


Figure 4. 7 Surface comparison of two proteins. At left Thc_Cut2 and at right Thc_Cut1 [25]

Finally, a study by Donelli et al. [26], showed the different results on degrading crystalline and amorphous PET using alkali (sodium hydroxide, NaOH) or enzymatic methods applying a cutinase from *Fusarium solani pisi,* which is a fungus.

Following the trend of Water Content Angle (WCA) it was discovered that alkali treatment was more efficient than the enzymatic since it increased the hydrophilicity of PET films, especially on amorphous PET [26], as it can be seen in figure 4.9.

Moreover, thanks to Fourier transform-infrared spectroscopy (FTIR) indexes it was known that both treatments induced structural and conformational rearrangements, but in a different way: crystalline PET was modified more by alkali treatment, while for the amorphous one, cutinase application was more effective [26]. The lower activity of cutinase towards crystalline PET was due to hindrance since the PET chain segments were blocked in a rigid structure and thus it was more difficult for them to enter the active site [26]. On the other hand, amorphous PET degradation was favoured by cutinase because the enzymes were able to work also in the bulk of the material, whereas alkali were generally working just on the surface [26].

This confirms that not only in PLA degradation, but also in PET hydrolysis the crystallinity degree is one of the most important parameters that must be taken into account when choosing the treatment.

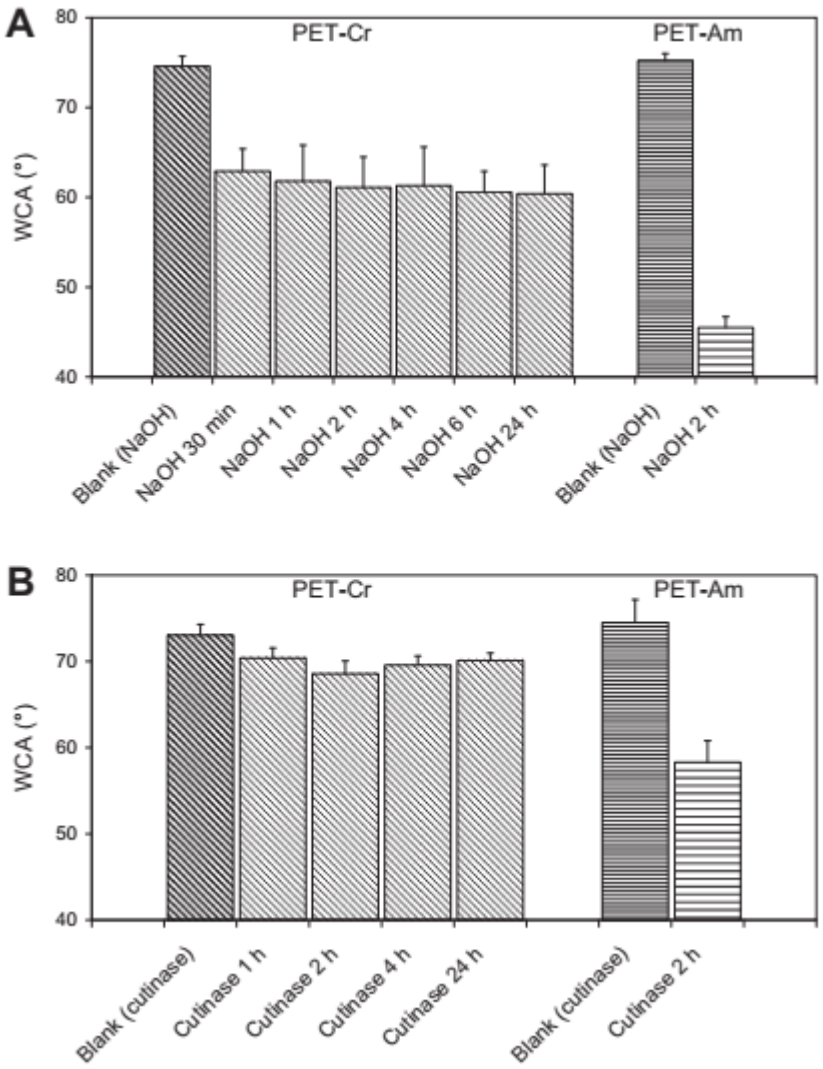


Figure 4. 8 WCA values of PET-Cr and PET-Am films subjected to alkali (A) or enzymatic method (B) [26]

4.4.3 Modified cutinases

As well as for PETase, biochemical modifications have been proposed also for cutinases, to get a more attached substrate and create more space in the active site [24]. Chen et al. [24] achieve these aims by replacing some residues in the binding region and substituting others with smaller ones in the active site.

Moreover, to increase the action of cutinases towards PET degradation especially, they inserted some binding domains to [24]:

1. Increase the amount of active enzyme on the polymer interface
2. Partially disrupt the structure of the polymer and make it more accessible for the catalytic domain.

4.5 Leaf and Branch Compost cutinase LCC

The cutinase used in this experiment did not come from a specific microorganism: it can be found in a mixture of leaves and branches (and from that the name, LCC leaf-branch compost cutinase) cut from trees, after they have been treated with urea and agitated [27]. The sequence of that enzyme was provided by Sulaiman et al. [27], as part of a research

they led in order to build a DNA library of genes encoding for for lipases and esterases in leaf and branch compost, and it showed an identity of 57.4% to *Thermobifida fusca* cutinase. LCC has the same catalytic triad and oxyanion hole of the other cutinases.

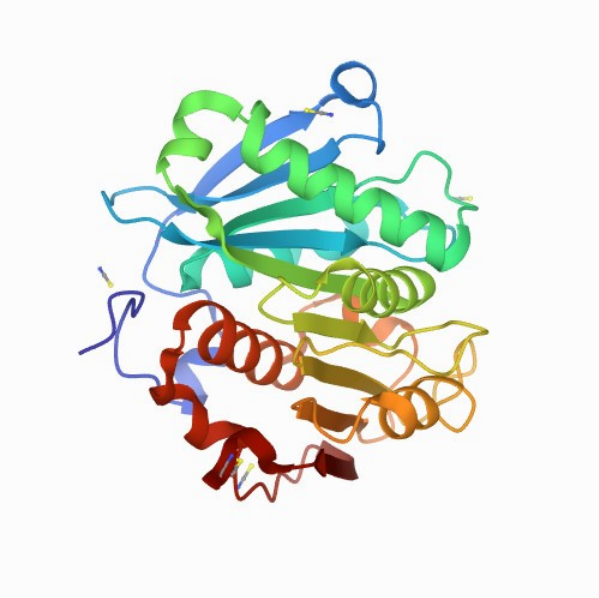


Figure 4. 9 Crystal structure of LCC homolog, called 4eb0, deposited in RCSB PDB by Sulamain, S., You, D.J., Eiko, K., Koga, Y., Kanaya, S.

This enzyme presented the highest activity at pH 8.5 and 50 °C, slightly lower than the cutinase from *T. fusca* (pH 8, but 60 °C), but still higher than the operational conditions of PETase.

4.6 Differences between PETase and cutinase

The studies described reveal that cutinases can play a role in PET degradation, so they must have something in common with PETase. Eventually, PETase and bacterial cutinase share some aspects of the chemical structure, especially for the sequence identity and the active site [19], visible in figure 4.10 (A), (B), (G) and (H). PETase is like cutinase from *Thermobifida fusca* (DALI server score is 42.4), *Saccharomonosphora viridis* (42.3) and *Thermobifida alba* (42.1) [20]. However, they have some differences, and these are changing the rate of the degradation and, sometimes, also the released products.

PETase surface, figure 4.10 (C), has an isoelectric point of 9.6 and it is a dipole; *T. fusca* cutinase surface has a neutral pI of 6.3, figure 4.10 (D), due to the homogenous distribution of acidic and basic residues [17]. This results in changed attraction for the substrates: PETase tends to attract more charged molecules, while cutinase more neutral ones.

Furthermore, PETase has an active site that accommodates better aromatic compounds, visible in figure 4.10 (E), perfect to face the highly hindered benzene rings of PET and, more generally, every semi aromatic polyester [17]. This is due to a change in amino acids: serine, in PETase, and phenylalanine, in cutinase, which active site is shown in figure 4.10 (F), in the lining of the active-site cavity [17].

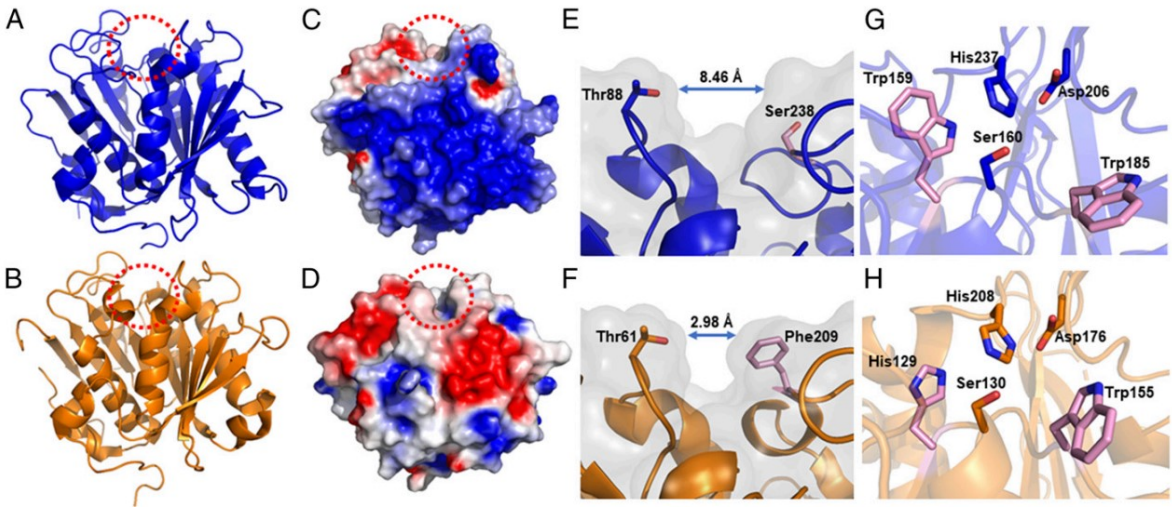


Figure 4. 10 Comparison of the structure of PETase and *Thermobifida fusca* cutinase. (A) PETase and (B) *T. fusca* cutinase cartoon representation at 0.92 Å resolution, the active site is circled in red. (C) Electrostatic Potential Distribution of PETase and (D) of *T. fusca* cutinase, going from red (acidic) at -7 kT/e to blue (basic) at 7 kT/e (k Boltzmann´s constant, T temperature and e charge of the electron). (E) View of the active site of PETase and (F) *T. fusca* cutinase (G) Close-up view of the PETase and (H) *T. fusca* cutinase active site [17]

Joo et al. [19] performed another comparative study on the active site between PETase and cutinase from *Thermobifida fusca.* The catalytic triad was found to be the same in both enzymes, constituted by Ser-His-Asp in same positions, fig. 4.11 (A), so the catalytic mechanism remained the same [19]. They had the same residues in subsite I, fig. 4.11 (A), thus the first MHET moiety bonded in the same way [19]. Anyway, subsite II, fig. 4.11 (A), had different conformations: His169 and Phe249 of the cutinase were replaced by Trp159 and Ser239 in PETase [19]. This change in the second enzyme increased the activity towards BHET and PET [19]. The connecting loop of β8-α6 was also different, fig. 4.11 (A): PETase had an extended loop because it had three extra residues (Asn244, Ser245 and Asn246) and this formed a continuous cleft from subsite IIa to subsite IIc [19].

Anyway, the biggest difference between those enzymes remained the existence, for PETase, of two disulphide bonds, rather than just one [19]. The common one, fig. 4.11 (D), was located in the same region (Cys281 and Cys299 in TfCut2; between Cys287 and Cys302 in the cutinase; between Cys276 and Cys294 in TaCut and between Cys273 and Cys289 in PETase) but it did not influence the active site, rather it stabilized the enzyme structure [19]. The additional disulphide bond for PETase, fig. 4.11 (E), on the other hand, was formed between Cys203 and Cys239, near the active site, where all the other enzymes had Ala residues, fig. 4.11 (F) [19]. This led to an increased thermal stability, as the experiments suggested [19].

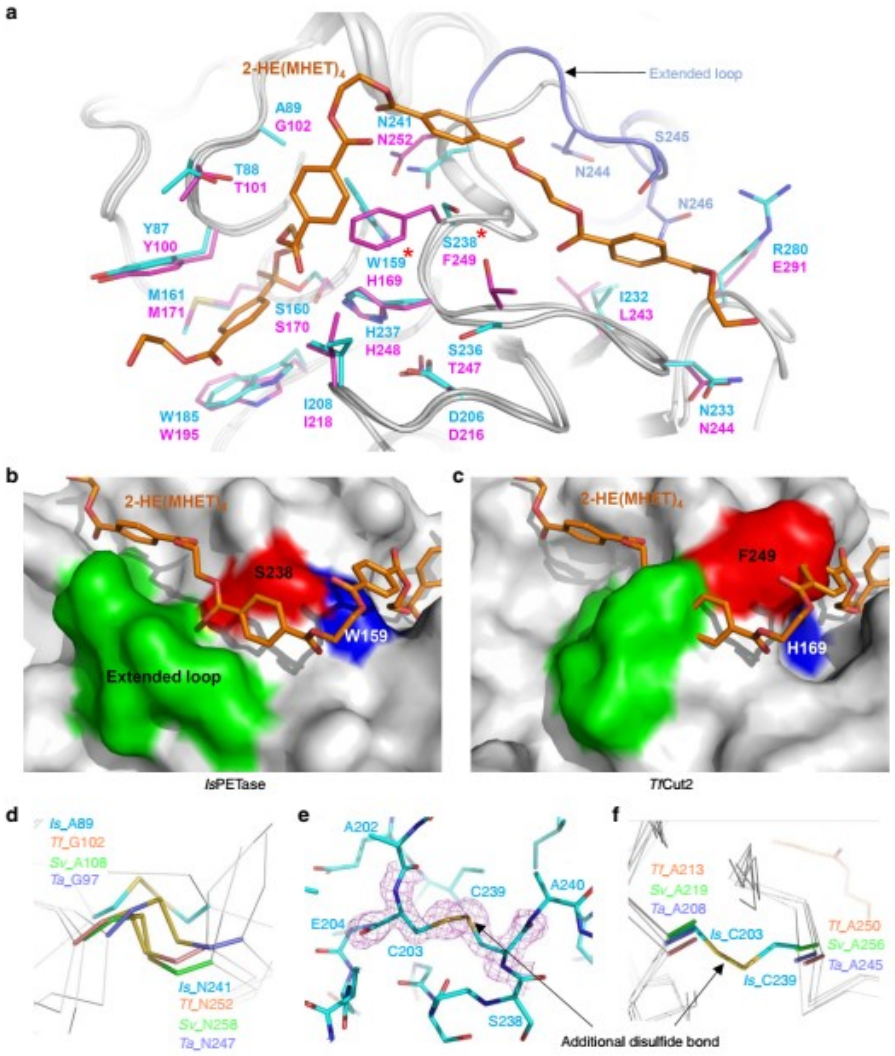


Figure 4. 11 Structural comparison of PETase and *T. fusca* cutinase. (A) Their superposition. (B,C) Differences on subsite II on PETase (B) and cutinase (C). (D) Disulfide bond found in all PET degrading enzymes. (E) Additional disulfide bond region in PETase compared to the same region in other PET degrading enzyme. (F) Residues forming the additional disulphide bond in PETase. [19]

4.7 Experimental bio-polyesters

In the second chapter an overview of the common bioplastics' production processes and properties has been provided. The common property that makes most of them easily biodegradable in the environment is the ester bond. So, from a theorical point of view, other polymers could be biodegradable as well, if they possess this, or other easily reacting, type of bond. Moreover, if these polymers are produced from waste residues the circular economy can be improved. This is the reason why this research will manly focus on polymers produced from biomass fermentation, forestry residues or as part of some microbial metabolism final product or intermediate.

The investigated bio-polyesters are either heteropolymers or copolymers, aliphatic or semi-aromatic; thus, they are quite different one to another. Despite this, the building blocks are the same, they were just joined in different ways and ratios, and they are diols and organic acids.

As organic acids, maleic acid (monomer A), figure 4.12, and itaconic acid (monomer B), 4.13 were used.

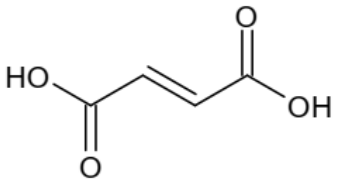


Figure 4. 12 Monomer A chemical structure

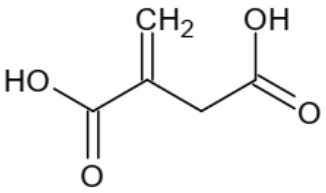


Figure 4. 13 Monomer B chemical structure

Maleic acid has normally a petroleum origin, coming from butane or benzene; however, it can be produced from the oxidation of renewable sugars through furan route, with furfural intermediate in the process [28]. Yu et al. showed [28] that furfural could have been produced from hemicellulose in systems using γ-valerolactone-H_2O and betainehydrochloride-H_2O as catalysts.

On the other hand, itaconic acid is of even easier bioproduction since is the final product of some fungal metabolism, such as *Aspergillus itaconicus* or *Aspergillus terreus*, which use glucose or sucrose as substrate, but also starch, molasses and corn syrup [29]. Then, itaconic acid is filtrated, evaporated, cooled down and crystallized [29].

The used diol, monomer D, figure 4.14, comes from a plant residues group, the terpenes, that are mainly found in the pine-tree resin turpentine [30]. Its structure is partly covered since it is object of a future patent.

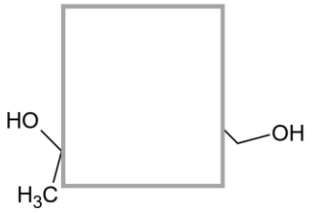


Figure 4. 14 Monomer D Chemical structure

The resulting heteropolymers are called P1 (D+A), figure 4.15.

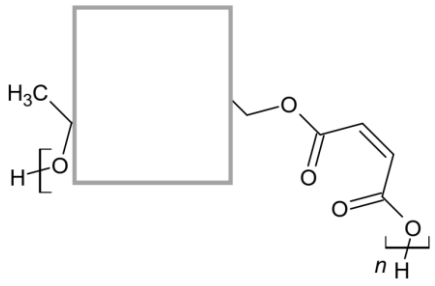


Figure 4. 15 P1 chemical structure

And P2 (D+B), figure 4.16.

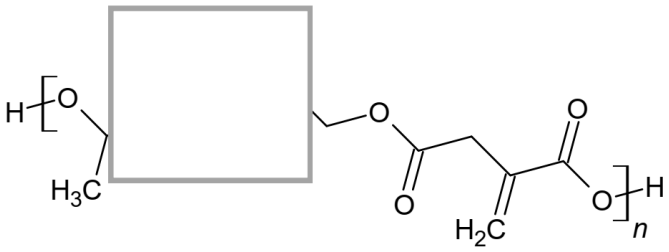


Figure 4. 16 P2 chemical structure

The other organic acid, involved this time only in the copolymers production, is furan-2,5-dicarboxylic acid (FDCA), figure 4.17 (monomer C).

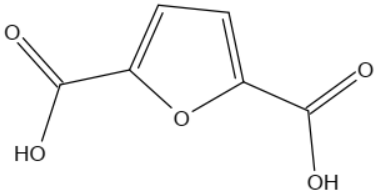


Figure 4. 17 Monomer C chemical structure

FDCA is a furan compound, it generally comes from oxidative dehydration of glucose with oxygen or oxidation of 5-hydroxymethylfurfural; bio-based FDCA can be produced via selective sugar dehydration, process that is still under investigation since the difficulty in controlling it [29].

The resulting copolymers structure, with the square and the circle again covering monomer D will be:

- P3: (D+A)+(D+C), figure 4.18.

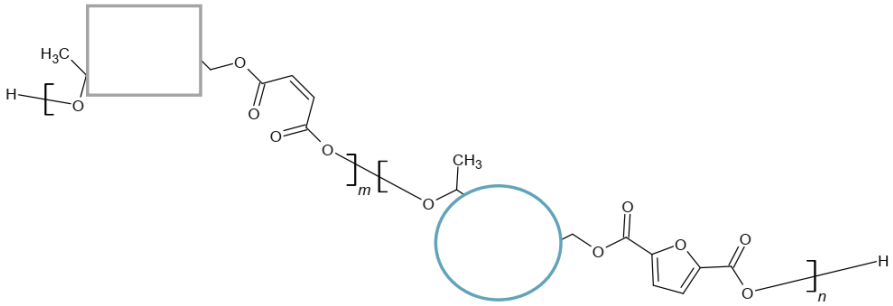


Figure 4. 18 P3 Chemical structure

- P4: (D+B)+(D+C), figure 4.19.

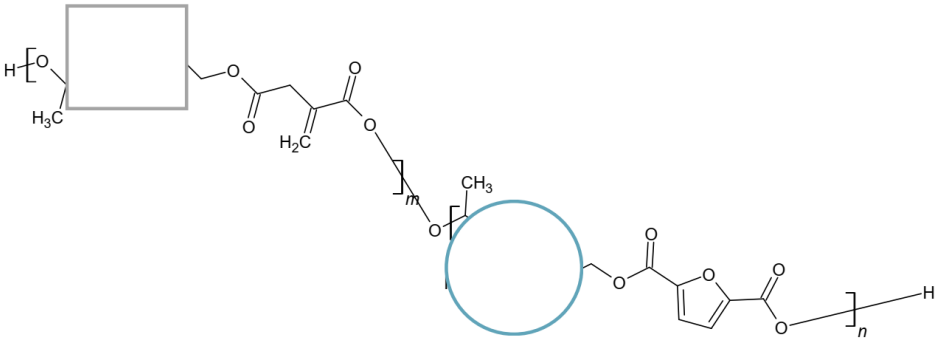


Figure 4. 19 P4 chemical structure

All polymers will be tested in their crosslinked form, with sulfur bridges between the blocks, as it can be seen in figure 4.20, which represents P2. They were thus assimilable to amorphous polymers.

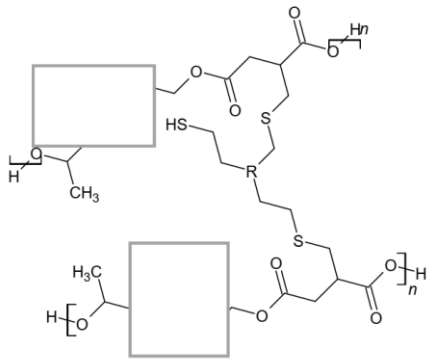


Figure 4. 20 Cross-linked version of P2

The theorical procedures followed to produce all of them will be now discussed, namely polycondensation, copolymerisation and crosslinking formation.

4.7.1 Polycondensation mechanism

Polycondensation is the polymerisation mechanism followed when a diol and a dicarboxylic organic acid are reacting in sequence. The mechanism consists in a polymerization before and, at the end, a polycondensation step [31].

The description of the process can be done in three approaches: considering the molecular species, the most complex one, or the functional group or, more easily, the overall reaction [31].

As this research will not focus on the production of the polymers, just the functional group analysis will be briefly explained, leaving a deeper insight to the reader.

Considering the functional groups, the reaction can be simply viewed as an esterification reaction between a carboxyl (called SA) and a hydroxyl (called G, glycols), producing five oligomeric segments (tSA, tG, bSA, bG and bDG) and water (W), with "b" standing for bound monomeric repeating unit and "t" for terminal functional group, as it can be seen in the next reactions [31].

$$SA + G \leftrightarrow tSA + tG + W \quad (4.3)$$
$$tSA + G \leftrightarrow bSA + tG + W \quad (4.4)$$
$$SA + tG \leftrightarrow tSA + bG + W \quad (4.5)$$
$$tSA + tG \leftrightarrow bSA + bG + W \quad (4.6)$$

Finally, the polymerisation has an end in the polycondensation step [31]:

$$tG + tG \leftrightarrow bG + G \quad (4.7)$$

There is also a side reaction producing diglycol repeating units [31]:

$$tG + tG \leftrightarrow bDG + W \quad (4.8)$$

In this research, P1 and P2 are the two heteropolymers produced.

4.7.2 Copolymerisation of diols and dicarboxylic acids

For the copolymerisation mechanism, the process consists of three steps [32]:

1. Polymerization of polymer 1 starting with monomer 1 and capping of the propagating end
2. Isolation and dissolution in a solvent of polymer 1 plus addition of monomer 2
3. Reaction to transform the functionalized ends into propagating species which will polymerize monomer 2.

The propagation mechanism, both in stage one and three can be carried out following four propagation mechanisms: anionic, cationic, free radical and Ziegler-Natta [32].

In this case the followed procedure was even more complicated, since the monomers were three (A, D and C or B, D and C): however, the mechanism remains the same, adding the blocks one a time.

4.7.3 Crosslinking

The crosslinks of the polymers were done through a photocrosslinking process, using UV light and an initiator, with a photoactive agent called Trimethylolpropane tris(3-mercaptopropionate) TMTP, visible in figure 4.21.

This photoactive agent can absorb the light energy, in the UV-visible spectral region, between 250 and 450 nm [33]. This energy is used to create reactive intermediates, free radical, reactive cations, nitrenes, etc, from which the polymerization or the crosslinking begins [33]. However, the polymers themselves must contain a chromophore group, to create the chemical bond [33].

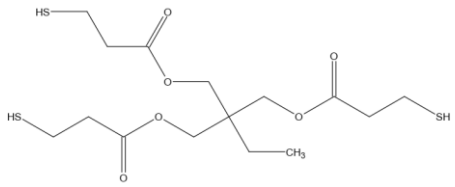


Figure 4. 21 Photoactive agent, TMTP

4.8 Possible PETase and LCC degradation mechanism on studied bioplastics

PETase and LCC cutinase will be the applied enzymes in this research. Even though they have been tested mainly on PET and other known polyesters, they could hydrolase the ester bond between the monomers, although the degradability depends also on hydrophilicity-hydrophobicity balance, crystallinity, orientation, and other morphological properties [34].

The issue, however, lies in the fact that just two out of four polymers are semi aromatic, P3 and P4, so the ability of the enzymes and especially of PETase, very high towards polymers with a benzene ring, could be compromised. Moreover, the polymer must be flexible enough to enter the enzyme active site and it must possess charges able to bond with the ones on the enzyme surface. In general, aromatic chains are more difficult to bond because they are less flexible and with a particular electrostatic configuration and so their enzymatic degradation is slower [35], but this is the reason why PETase and cutinase, with their very specific structure and residues sequence, act well towards them.

Finally, it must be considered that all of them were provided in the crosslinked form, which guarantees the polymers to be amorphous, but it can make it more difficult the hydrolysis, since the enzymes are not able to break sulphur bonds.

4.8.1 Aliphatic heteropolymers

P1 and P2 are aliphatic heteropolymers; as reported by Austin et al. [17], in their experiments PETase was able to depolymerize other aromatic polyesters, such as PEF (Polyethylene 2,5-furandicarboxylate), but not aliphatic ones, like PLA and PBS (polybutylene succinate). Thus, a degradation of P1 and P2 with PETase is not expected to occur.

Considering the cutinase, however, the hydrolysis probability is slightly higher since it catalyses many different reactions, also involving aliphatic molecules. The probable mechanism, for both P1 and P2, will probably form first intermediated, such as the dimer, and then, if the reaction proceeds, also the monomers, as it can be seen in figures 4.22 and 4.23.

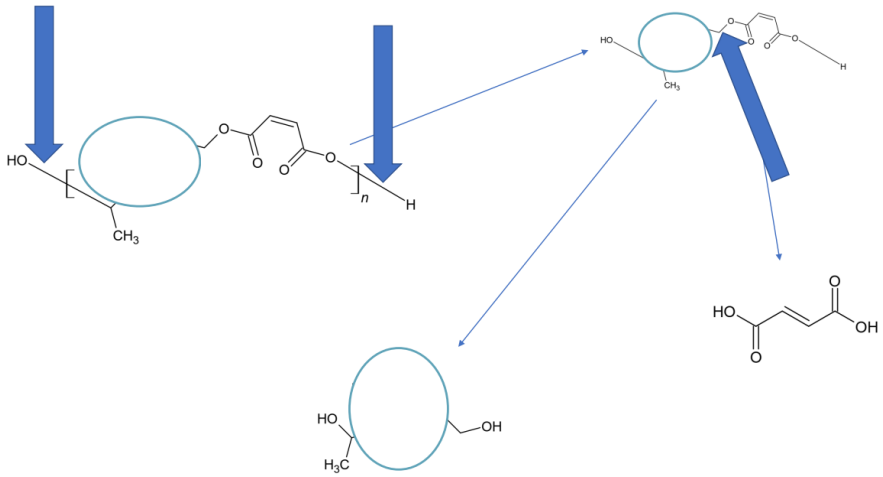


Figure 4. 22 Possible degradation mechanism of P1, blue big arrows indicating the cleavage sites

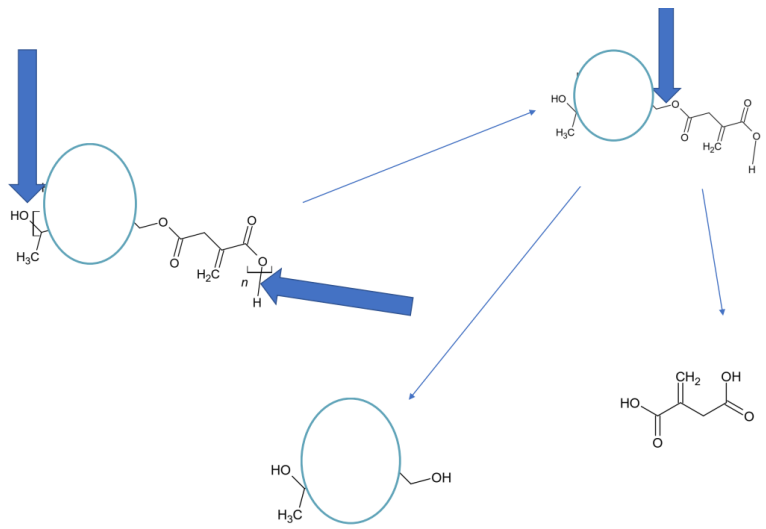


Figure 4. 23 Possible degradation mechanism of P2, blue big arrows indicating the cleavage sites

4.8.2 Semi aromatic copolymers

In this case monomer C, which has an aromatic ring in the form of furan will probably make PETase the best option for its active site more prone to face benzene ring compared to the cutinase one. However, it is interesting to consider the comparative study by Yoshida et al. reported in the paper written by Shirke et al. [36]. The results showed that although PETase was the most active for PET hydrolysis at ambient temperature, LCC cutinase outperformed it at high temperatures [36]; so, in this case, the operative conditions could change the results.

Considering the degradation steps, intermediates, as dimers, will be formed as well as monomers, figure 4.24 and 4.25. However, applying just PETase and not MHETase will probably shift the results towards the dimers.

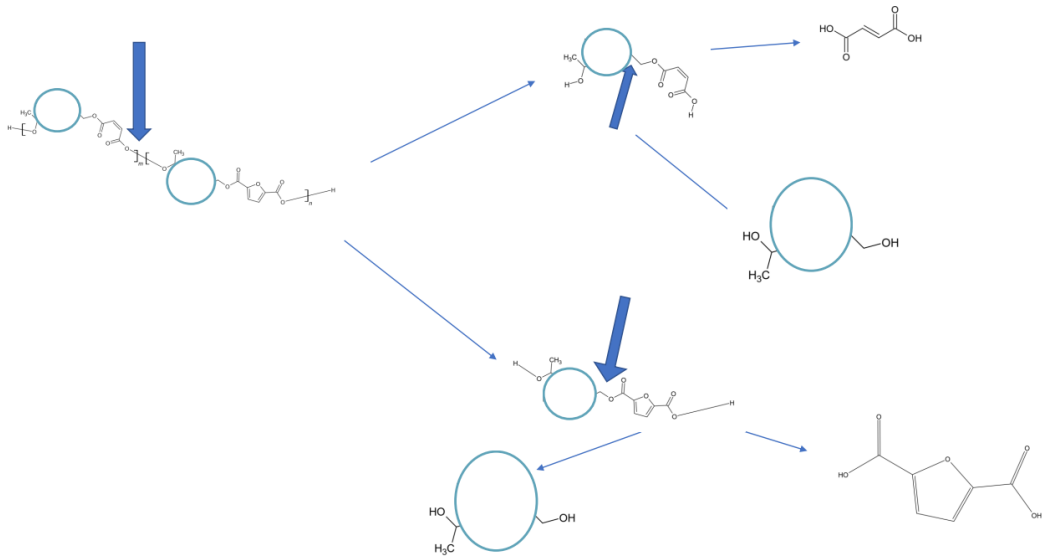


Figure 4. 24 Possible degradation mechanism of P3, blue big arrows indicating the cleavage sites

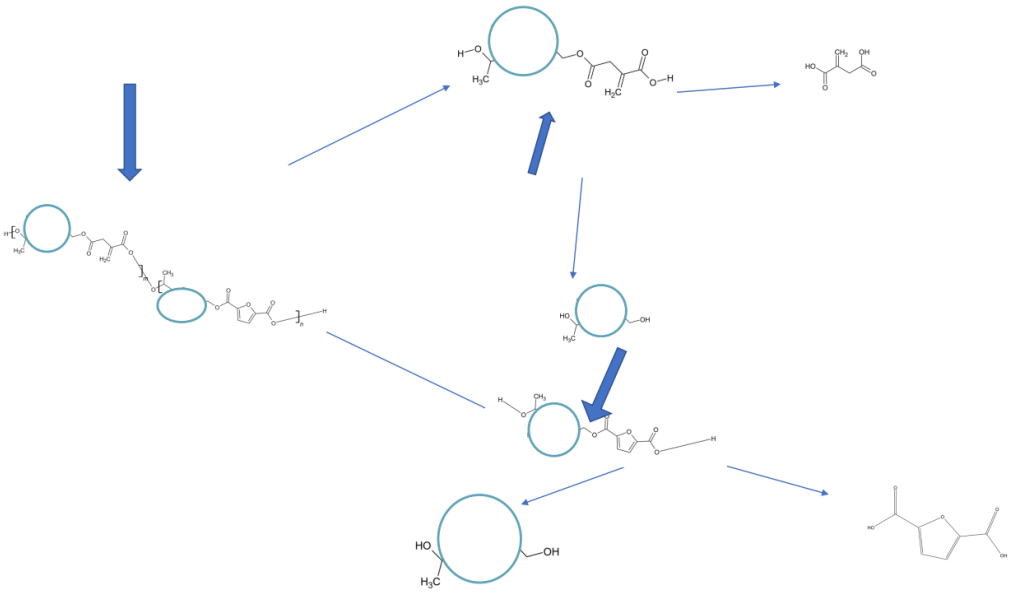


Figure 4. 25 Possible degradation mechanism of P4, blue big arrows indicating the cleavage sites

4.8.3 Crosslinked form

Crosslinking results in a decrease in crystallinity and higher rigidity [37]. The more the amorphous the polymer, the better the enzymatic degradation, ad Donelli [26] reported. Despite that, the problem here remains the presence of sulfur bridges. As a matter of fact, a solution could be of not only break the ester bond, but also the sulfur bonds linking, a process generally called “devulcanization”.

Carbon-sulfur bonds are difficult to break, since they are chemically- and thermostable; thus, several studies, however not on polyesters, have been carried out in the last few years to investigate how they can be degraded. One promising possibility follows the conclusion of Edwards et al. [37]. They analysed the mechanical and mechanochemical methods to dissolve the crosslinks in ground tire rubber (GTR) (crosslink density of $117*10^{-6}$ mol/g). The mechanical devulcanization was carried out in the extruder at varying temperatures, from 175 to 275 °C, and screw speeds, from 30 to 80 rpm. For the mechanochemical method, the same instrument was adopted, with a range of temperatures from 150 to 220°C, applying DPDS (Dipropyl Disulfide) concentrations of 5 to 30 g /kg GTR and a constant speed of 30 rpm. The results showed that increasing the temperature in the extruder resulted in both cases in a strong decrease of the crosslink density, whereas DPDS and extruder speed did not vary it at the same level [37]. However, investigating the selectivity towards the crosslink scission rather than the C-C cleavage, mechanochemical methods seems to be the most promising technique, especially when DPDS is coupled with process oil [37].

However, this range of temperature will not be applied in this study and, more generally, seems impossible to couple with enzymes, which have strict operational range of temperatures, even when genetic engineering is applied. That said, the results suggested also that the crosslink could be overcome applying some chemicals. Once the sulfur bridges are broken, the simple polymers can be found, and they will follow the degradation pathway described above.

5. Enzymes production

5.1 Plasmids

Producing enzymes in a laboratory is a well-established technique centred on the use of plasmids. Plasmids are defined as "autonomous self-replicating molecules of DNA" [38] and can be found in many cells, called hosts. As the definition suggests, their important characteristics is that they are independent from the genetic information of the cell for two reasons [38]:

1. The same plasmid could be found in cells of different species
2. The plasmid could be present or not in a cell, thus they are not a constant in the cell genetic heritage, and they are not needed for the microorganism to growth

Their DNA is assembled forming a circle and its dimensions vary from half a dozen to several hundred genes [38]. They can transfer from one cell to another in two ways: moving directly, if they possess the transferability, typical of medium/big plasmids, or they can be mobilized by self-transferable plasmids [38]. Anyway, plasmids belonging to the same family, thus, sharing very similar sequences in their partition and replication genes, cannot survive in the same cell; a property called incompatibility [38].

The property that makes them autonomous is the capacity of self-replicating, since they possess an origin of replication, where the synthesis begins, figure 5.1 [38]. However, they do not have genes to produce energy, raw materials and carry other enzymes activities in their genetic heritage [38]. Plasmids are, thus, similar to viruses, with the difference being the total absence of protein coats in the firsts, which is the reason why they cannot survive outside the host cell and, thus, they tend not to kill it since they need it [38].

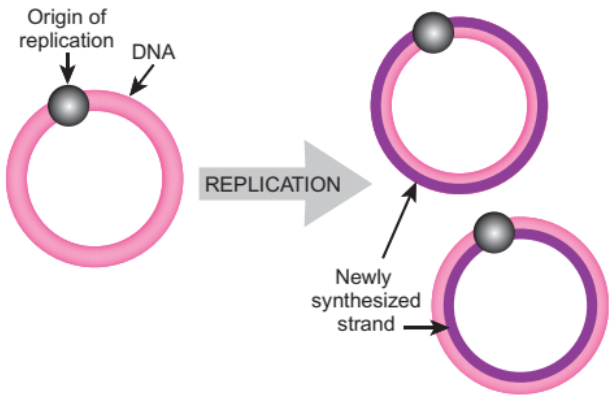


Figure 5. 1 Plasmids as rings of double stranded DNA [38]

Plasmids DNA replicates by two methods: bidirectional replication or rolling circle [38].

In the first case, in figure 5.2 left, DNA opens in the replication origin and from there two replication forks move in opposite directions around the circular DNA until they meet [38]. In the second case, figure 5.2 right, one strand of the double-stranded DNA remains still, while the other starts to roll away remaining circular: DNA starts to be synthesized in the broken strand, with the circular one used as a template, filling the gap between them; then another piece is rolled and filled and so on [38]. At the end, the original broken strand is unrolled, and the circular strand is paired with a new strand [38]. Then, how the single strain

is paired depends on the circumstances [38]. If the plasmid has a simple replication, then the single strand is used as a template and another strand is produced; the double segment is cut free and then circularized [38].

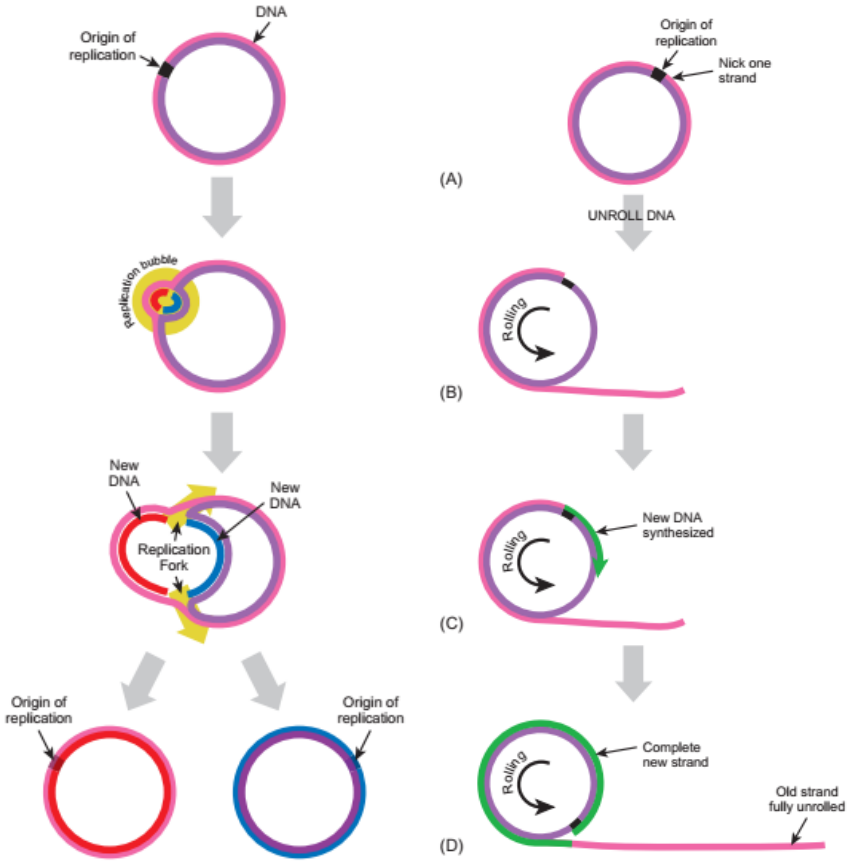


Figure 5. 2 On the left, bidirectional Plasmid Replication; on the right rolling Circle Replication [38]

If plasmids can transfer themselves, they have two origins of replication. Thus, they use the first method, bidirectional replication, when their host cell divides; while they choose the other option when they are moving to another cell during conjugation [38]. These two processes start in different replication origins. Once the copies are produced, they are partied among the cells through a system that uses protein filaments part of the cytoskeleton, which bind to the plasmid through a recognition site [38].

Apart from the origin of replication, a plasmid contains other information, such as genes for proteins production, but also for its optimization and induction. The ready-to-use plasmids available on the market have empty spaces where the sequence codifying for the wanted protein can be added. To make the host cell, where the plasmid will be inserted, able to transcript the gene for this protein, a promoter, defined as a sequence located upstream the ribosome-binding site of the plasmid, is required [39]. To have a transient induction, the promoter is repressed at the beginning and then induced by an inducer addition [39]. The most used promoters are lactose (*lac*) and tryptophane (*trp*); they can also be combined to create the hybrids *tac* and *trc* [39]. Less used, but still important, are the phage lambda promoters, the phage T7 promoter (T7), and the alkaline phosphatase promoter (*phoA*) [39].

In case of lac system, in presence of lactose or the inducer isopropyl-β-D-thiogalactoside (IPTG), the lac repressor changes conformation and cannot bind the lac operator; this allows the RNA polymerase to bind the lac promoter and begin the transcription [39].

Another sequence present in the plasmid is the antibiotic resistance one, useful to understand which of the host cells have incorporated the plasmid inside them. Thus, this sequence is a marker: when the antibiotic is introduced in the growth medium just the cells with this gene, and thus with the plasmid inserted, will survive [40].

Finally, there are some sequence in the plasmid, called tags, which are useful to detect the protein in its final localization since the residues involved will bind the molecule immobilized in a column being recovered then [39]. One of the most widely used fusion tags is a sequence of histidine amino acids, 6 to 10, the so-called His-Tag, able to bond with nickel ions [39].

5.2 Host properties towards plasmids and transformation

Once the plasmid is prepared, it is transferred in the host. In this case *Escherichia coli* will be the one chosen; thus, a short description on its characteristics and how to insert the plasmid into it will be provided.

Escherichia coli is a rod-shaped Gram-negative bacterium with a circular genome of 4.6 Mb [41]. Nowadays, its usage for propagation, manipulation and characterization of recombinant DNA is well established and it can grow on chemically defined media rapidly [41].

Once the host has been chosen, the vector should be inserted into it. This procedure is generally known as transformation, while "the ability of transform or accept those external DNA" is called competence [42]. Natural competence is something that very few bacteria have, thus some methods to induce it have been studied, generally divided into electroporation and chemical transformation methods [42].These results in open gated membranes channels and the plasmid enter through them; advantages of using chemical transformation are ease, relative efficiency and lack of need for an apparatus, such as an electroporator [42].

However, electroporation is probably the most efficient and reliable method for transformation into *E. coli* using plasmids [43]. It consists in an electrical pulse, which polarizes the membrane and forms transient pores that can be used by the DNA to enter the cell [43].

Apart from electroporation and chemical methods, there is another way of transforming plasmids into a host: bacterial conjugation, "the contact-dependent transmission of genetic information from a donor bacterium to a recipient cell" [44]. Some plasmids, such as fertility ones, are self-transmissible, meaning that they can move from one cell to another through a pilus [44]. Other plasmids are mobilizable, but not self-transmissible, thus, they need necessary functions [44].

5.3 Proteins extraction

Once the plasmid is inserted in the host, the protein production starts and recombinant products are released; their final localization may influence its production and its tertiary structure, because the post-translation modifications happen in different places in the cell. Recombinant proteins exist in three compartments: cytoplasm, periplasm or extracellular medium [39].

Cytoplasm is a reducing environment; thus, soluble proteins may be directly extracted from the supernatant of lysed cells [39]. Insoluble proteins generally tend to aggregate in

inclusion bodies, which are desirable since they are easily isolated through centrifugation, but also they protect the protein from proteolytic degradation, and toxic proteins, in the inactive state in this case, are more tolerated [39]. However, these insoluble proteins in the inclusion bodies may need to be refolded [39].

The periplasm is an oxidizing environment and it generally contains enzymes that catalyse the formation of disulfide bonds [39]. Anyway, the protein transfer from cytoplasm to periplasm may lead to lower expression levels and a change in their conformation: some proteins may be degraded before doing so, because they remain stuck in this passage [39]. Moreover, extracting proteins from the periplasm involves an osmotic shock usage. [39]

If the proteins are found in the extracellular medium it means that they have been secreted, which is generally advantageous [39]. In this way there is less proteolytic activity, *E. coli* secretes very few proteins; purification is simplified, and the protein is likely to be soluble and in its native conformation. [39]

Once the proteins are separated from the cell, either by lysis or by recovering from the external medium, they must be purified one from each other. The separation and purification techniques can be many and, generally, not only one is used. Among the most widely used procedures anion and cation exchange chromatography, hydrophobic interaction chromatography, gel filtration and affinity chromatography are found. In these cases, the separation happens due to different molecular weight, charges, or interaction with a solvent. However, the choice of the best procedure, both considering the request technique and the order of usage of those, may be complicated and, sometimes, can lead to a low purity of the target protein. Sometimes, to check whether the target protein has really been expressed in the host, SDS PAGE (sodium dodecyl sulphate, polyacrylamide gel electrophoresis) is used. This is a separation method that divided the proteins depending on their molecular weight and charge: comparing the results with the one of a host cell without the target protein is a way to find out if the transformation and expression were done correctly.

6.Materials and methods

6.1 Materials

All the materials used in the experiment are listed in table 6.1.

Table 6. 1 List of used materials

Name	Composition	Supplier
CD43 Recombinant Protein	*Escherichia Coli* cell for peptides expression	Sigma
Tryptone	Peptides digested by trypsin from casein	VWR Life Science
Yeast Extract	Extract of the yeast	VWR Life Science
Sodium Chloride	Salt	M
Ampicillin	Antibiotic	Sigma
IPTG	Inducer	AppliChem Panreac
B-PER™ Complete, Bacterial Protein Extraction Reagent	Nonionic detergent-based solution	Thermo Scientific
See Blue Standard Plus 2	Protein (4-250 Da) mixture used as standard marker for SDS-PAGE	Thermo Scientific
Instant Blue™	Solution stain (Coomassie dye, ethanol, phosphoric acid, and solubilizing agents in water)	Expedeon
4-15% Mini-PROTEAN® TGX™ STAIN,15 well comb 15	Polyacrylamide protein Gels for SDS-PAGE	BIO-RAD
Sample Buffer, Laemmli 2* concentrate	4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl	Sigma
Ni-NTA Agarose	Nickel-nitriloacetic acid coupled with Sepharose Cl-6B support	Qiagen
Tris	Tris (hydroxymethyl) aminomethane	VWR Life Science
HCl	Inorganic Acid	Sigma Aldrich
Imidazole	Aromatic compound	Sigma Aldrich
PD-10 Desalting Columns Sephadex™ G-25 Medium	Prepacked columns containing Sephadex G-25 medium	GE Healthcare
Bio-Rad Protein Assay Dye Reagent Concentrate	Kit containing bovine serum albumin or bovine γ-globulin standard	BIO-RAD
Triton X-100	Nonionic surfactant	Sigma
Sodium Carbonate	Inorganic compound	M
Potassium Dihydrogen phosphate	Potassium salt	VWR
MQ- Water	Ultrapure (type 1) water	M
KOH	Inorganic compound	VWR
MeOH	Alcohol	VWR
Ethyl Acetate	Organic compound, ester	VWR

6.2 Methods

6.2.1 Enzyme preparation

Plasmid choice

Both enzymes were produced separately after being inserted in the plasmid pET-21b (+) from Invitrogen.

As it can be seen from figure 6.1, the plasmid had different sequence landmarks, among those it is important to remember:

- His-Tag, it consisted of at least six histidine (His) residues. It was used to further allow purification of the enzyme in a column, since histidine had the highest ability of coordinating metal ions on its surface.
- An origin of replication
- An inducer, a sequence useful to disable repressors and binding activators.

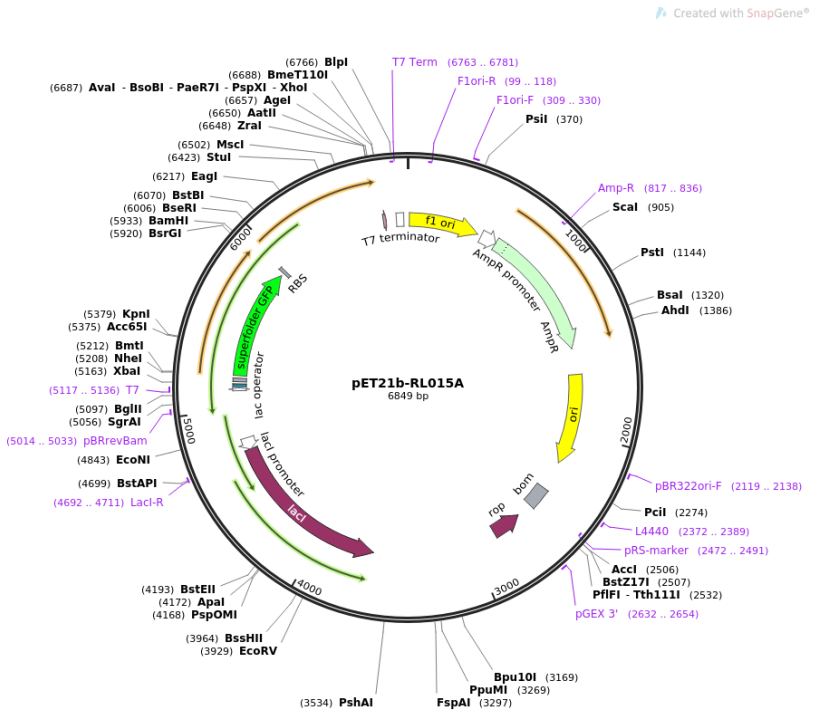


Figure 6. 1 Representation of pET-21b (+) sequence from Addgene

The genes responsible for codifying PETase (Wild type) and cutinase (LCC, the same described in paragraph 4.5), were coming from Invitrogen as well. They were amplified through a polymerase chain reaction (PCR) with KOD hotstar polymerase, following a procedure constituted by a cycle repeated 30 times:

- 70 °C for 20 s
- 55 °C for 10 s
- 95 °C for 20 s
- 95 °C for 2 minutes

The plasmid and the genes were separately unified with the HiFi DNA assembly at 50 °C for 1 hour. Finally, the produced plasmid carrying the esterases genes was amplified using

another primer, codon pBRev and T7 terminal, already present on the pET21 sequence. The final sequence for LCC were:

LCC_PET21_F	agcaaatgggtcgggatccgGAATTCATGAGCAATCCG
LCC_PET21_RV	ggtgctcgagtgcggccgcaAAGCTTTTAATGATGGTGG

Plasmid insertion in the host

The plasmids were inserted in Escherichia coli LB 21 through heat shock transformation:

1. 5 µl of the plasmid were mixed with 50 µl of XL-I blue cell
2. Everything was put on ice for 30 minutes
3. Everything was put at 42 °C for 1 minute
4. Everything was put on ice for 2 minutes
5. 400 µl of 2 YT, a medium which concentration will be described next, were added
6. Everything was put at 37 °C and 400 rpm for 1 hour in the incubator, Infors HT
7. 200 µl of the mixture were added on an agar plate
8. The Amp plate was cultured at 37 °C overnight in an autoclave, Systec VX 150

Microorganism culture

After the cells had grown on the plate, they ned to be transferred in a flask and start the culture with a medium, which provided them with nutrients. The medium, 2YT, was prepared mixing, per 1000 ml:

- Tryptone 16 g
- Yeast extract 10 g
- Sodium chloride 5 g
- Ampicillin, 1 ml

Then, a preculture, to start the growing of the cell, was done: 2.5 ml each of medium 2YT were transferred in 50 ml capacity Falcon tubes and then the cells were withdrawn and posed inside of them. They were left partly opened, to let the oxygen going in and then put in the incubator at 37°C and cultured.

After approximately 20 hours, 200 µl of the cell were mixed with 1.8 ml of 2YT medium and their optical density was measured in the spectrophotometer V-1200 by VWR. This measure was related to the cell content and it was necessary since the inducer for the protein expression must be inserted just at a right concentration, with an OD of 0.55 or 0.65. Then, the total content of the Falcon tubes was reversed in 100 ml of medium in a 500 ml flask. Everything was incubated at 37 °C and 200 rpm until they reached the wanted OD.

Finally, the IPTG 1 M was mixed to reach a concentration of 1 mM, so 100 µl in each were inserted and the flasks were incubated overnight at 160 rpm and different temperatures for the two enzymes. Wild-PETase was cultivated at 20 °C, following all the studies presented before. LCC incubation temperature was first set to 37 °C, as Soulamain et al. [33] did. However, this operational condition did not give any expression, so 18 °C were chosen, similarly to PETase, and, eventually, LCC was produced.

Cell lysis

The 100 ml flasks were divided in 50 ml Falcon tubes and centrifugated in Sorvall ST 16R by Thermo Scientific at 4000 rpm, 4°C for 30 minutes. Then the liquid part was removed, and the pellet were left in the tubes and disposed on ice.

To lyse the cell once the enzyme had been produced, a chemical extraction was performed using B-PER. 3 ml of the buffer were put in each Falcon tube and they were mixed and put in the incubator at 25°C and 80 rpm for 20 minutes. Then they were finally centrifugated at

4000 rpm for 15 minutes. The following step depended on if either the protein ned to be checked for its expression or separated, which generally followed the check itself.

Enzyme expression

The expression of the protein was checked via SDS-PAGE, as it has been described in the previous chapter. To separate the proteins, they must have lost their 3D structure, which was done by adding a buffer, called Sample Buffer Laemmli 2x concentrate; 50 µl of the liquid from the Falcon tubes were mixed with other 50 µl of this buffer. They were heated up to 95°C for 5 minutes.

SDS-PAGE was performed in a commercially available gel, called Mini-PORETAN TGX STAIN FREE-GELS 4-15%. The used marker was See Blue Plus 2 Standard. The measure was performed adding 5 µl of the marker in the first position and 10 µl of the samples after that. The power supply by Bio RAD was set at 200 V (with fixed 400 mA) for 25 minutes, with the gel inserted in the chamber and covered with instant blue buffer. Then the gel was washed and disposed in the incubator overnight with Instant Blue.

Enzyme purification

To purify the enzymes two methods were used. PETase purification involved a machine called Äkta by Cytiva: it used a nickel column, which must have been washed for 10 minutes with ethanol and 10 minutes with milli-q water before starting the separation, to eliminate residues. Then a washing buffer, with 50 mM Tris-HCl (pH 7.4), 500 mM NaCl and 20 mM Imidazole, was run for 10 minutes at 1 ml/min. After that, with the same flowrate, the elution buffer, 50 mM Tris-HCl (pH 7.4), 500 mM NaCl and 300 mM imidazole, was run for 15 minutes. At this point the sample, with a volume of 20 ml, was injected, varying the percentage of elution buffer, from 50 to 100%.

LCC was separated thanks to nickel compounds, able to bound the histidine residues, as well, but this time those substances were not packed in a column but dissolved in a solvent called Ni NTA Agarose.

The procedure consisted in:

1. Inserting 4 ml of Ni NTA Agarose in 50 ml Falcon Tubes
2. Centrifuging them at 500 rpm for 30 seconds
3. Adding 10 ml each of buffer (50 mM TrisHCl, 500 mM NaCl, 5 mM Imidazole)
4. Centrifuging them at 500 rpm for 30 seconds to rinse them
5. Adding the sample
6. Stocking them in the fridge for 1 hour, shaking them every 15 minutes
7. Centrifuging them at 500 rpm for 30 seconds
8. Trashing the liquid
9. Adding 5 ml each of the same buffer of before, to wash them
10. Centrifuging them at 500 rpm for 30 seconds
11. Removing the liquid
12. Repeating steps 9, 10 and 11
13. Adding 5 ml each of another washing buffer (50 mM TrisHCl, 500 mM NaCl, 20 mM Imidazole)
14. Centrifuging them at 500 rpm for 30 seconds
15. Removing the liquid
16. Repeating steps 13, 14 and 15
17. Adding 3 ml each of the elution buffer (50 mM TrisHCl, 500 mM NaCl and 300 mM Imidazole)
18. Centrifuging them at 500 rpm for 30 seconds
19. Taking the liquid part

Enzyme desalting

Independently from the purification method, once the enzymes were separated from the other proteins they had to be desalted, using PD columns. Their top caps were removed, and the storage solution was poured off, then their end was cut. They were washed 5 times with 5 ml Tris 10 mM. 2.5 ml of the sample were injected, with further buffer if there was not enough amount from the separation, to fill up the bed completely. Finally, the column was eluted using 3.5 ml of Tris 10 mM and the desalted sample was collected.

Enzyme concentration

The enzymes were concentrated using Vivaspin® 20 centrifugal concentrator by Fischer Scientific: the samples coming from the desalting were poured in a diafiltration cup and Tris 10 mM was continuously inserted. The remaining salts and the eventual contaminants were separated thanks to the membrane.

Finally, to evaluate the concentration of the proteins, 10 µl of the sample were mixed with 200 µl of 5 times diluted Bio-Rad protein assay dye reagent concentrate. The measured was done using a plate reader, SPECTRAmax PLUS by Molecular Devices at 595 nm.

Nano differential scanning fluorimetry (DSF) of LCC

LCC is a more thermostable enzyme compared to PETase. Soulamain et al. [27] searched for the best temperature giving the highest enzyme activity and found it to be 50 °C, figure 6.2.

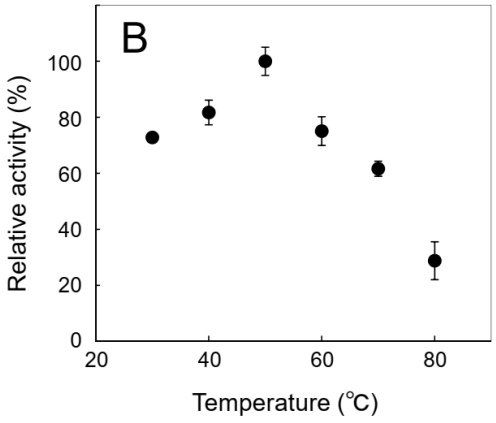


Figure 6. 2 Relative activity (%) dependency on temperature for LCC, determined in 10 mM sodium phosphate (pH 7.0) containing 2% acetonitrile using pNP-butyrate as a substrate [27]

To double check this information, the melting temperature of LCC was measured through the monitoring of tryptophan fluorescence in Prometheus NT. 48 nano DSF by Nano Temper technologies to understand at which temperature the unfolding was occurring.

6.2.2 Polymers degradation

To degrade the polymer, the samples from polymers P1, P2, P3 and P4 were cut, the weight table can be found in the appendix; then, they were subjected to different treatments.

In case of PETase application half of them was washed before and after the degradation to remove proteins (with Triton) and charges on the surface (with sodium carbonate) with the following procedure:

1. 1 ml Triton X-100 5 g/l was added in the sample vial
2. The vials were incubated for 30 minutes at 30 °C and 100 rpm
3. The liquid was removed, and 1 ml of Milli-q water was added
4. The vials were incubated for 30 minutes at 30 °C and 100 rpm
5. Step 3 and 4 were repeated other two times
6. The liquid was removed, and 1 ml of Sodium Carbonate 10.599 g/L was added
7. The vials were incubated for 30 minutes at 30 °C and 100 rpm
8. Steps 3 and 4 were repeated three times

While for LCC application all the samples were washed.

Then, the samples were mixed with the enzyme in different ratios:

1. 1 µg PETase per 5 mg plastic
2. 15 µg PETase per 5 mg plastic
3. 0 µg PETase, to have a reference sample
4. 1 µg LCC per 5 mg plastic
5. 15 µg LCC per 5 mg plastic
6. 0 µg LCC, as control

Finally, every vial was filled with a total of 1000 µl kpI buffer (M_W=119.98 g/mol; 1.7 g potassium dihydrogen phosphate, 200 ml MQ water, KOH to reach a pH equal to 7.2 and more MQ water to have a final volume of 250 ml) and everything was incubated at 30 °C and 100 rpm in INFORS HT Ecotron for PETase and 60 °C and 600 rpm in EPPENDORF Thermomixer for LCC both for 48 hours.

6.1.3 Products analysis

To characterize the samples, an HPLC model 1260 Quat Pump from Agilent Technologies was used. The mobile phase was composed by 0.1% trifluoracetic acid (TFA), acetonitrile (MeCN) 5-30% with a flow of 1 ml/min for 3 minutes, while the stationary phase, the column, was made of 3-phenyl 3 µm particles. 2 µl of the sample were injected. The detection occurred via UV and mass spectrometrum (MS) detectors, ES-API (electron spray-atmospheric pressure ionization) type.

Degradation occurred when monomers were detected in the supernatant of the vial, but to expect their peaks at the proper time a run of them and the buffer must have been performed.

Different ratios of the monomers on buffer, visible in the table in the appendix, were tested on the HPLC, after investigating their UV spectrum on the plate reader Spark® by Tecan3. They were all tested after being mixed with potassium phosphate buffer, but not monomer C, which was mixed with methanol, because it had a low solubility generally [45], and this chemical improves it.

The buffer with the product released was analysed in the HPLC, after a centrifugation in Heroeus Fresco 21 by Thermo Scientific at 7.2 rpm, after 24 hours and 48 hours. Finally, the samples with the best results were analysed also in the GC, QP2010 Ultra by Shimadzu, after being extracted with ethyl acetate. The carrier gas was helium, while the stationary phase was constituted by crossbond diphenyl dimethyl polysiloxane supported on fused silica, called Rxi-5ms columns by Restek. The temperature gradient was set from 70 °C to 230 °C in 10 minutes and then to 330 °C in 15 minutes. In case of PETase application the remaining films were washed, if they have been washed before, and subjected to SEM analysis with Hitachi S-4800 SEM.

7. Results and discussion

7.1 Protein expression and thermal degradation

LCC SDS-PAGE measurement, figure 7.1 on the square, confirmed that the protein was correctly inserted in the host and expressed, since the correct molecular weight row was only highlighted in the sample after being separated from the other products, third row, while the previous line, of the whole cell before separating the different produced proteins, first row, and the liquid after being washed but not purified, second row, did not show it. The other groups, not considered in the black square, referred to other culture conditions of the same LCC production and did not show that highlighted row in the purified supernatant.

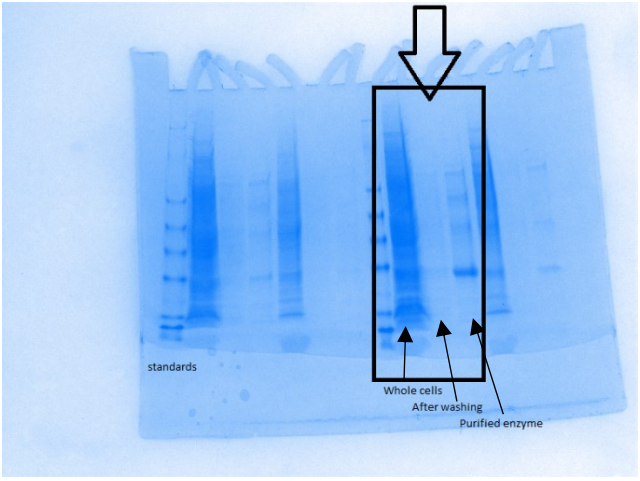


Figure 7. 1 SDS-PAGE analysis of LCC, the lines referring to different culture conditions

The melting temperature T_m analysis of LCC gave the following results, figure 7.2 and 7.3, in terms of ratio between the intensity at 350 nm and 330 nm and its first derivative. From both it is visible that 80 °C is the temperature at which the protein starts to unfold. Thus, it was decided to run the degradation at 60 °C, in order to have a margin before the protein lost its stability and still performing it at harder conditions compared to PETase, in order to take into account also that parameter.

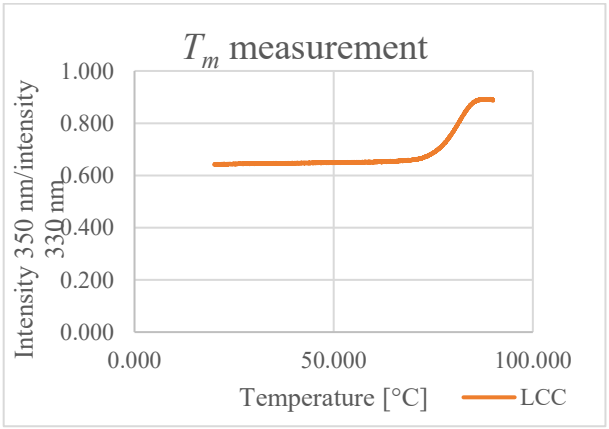


Figure 7. 2 *Tm* measurement of LCC express as ratio between intensity at 350 nm and 330 nm varying the temperature

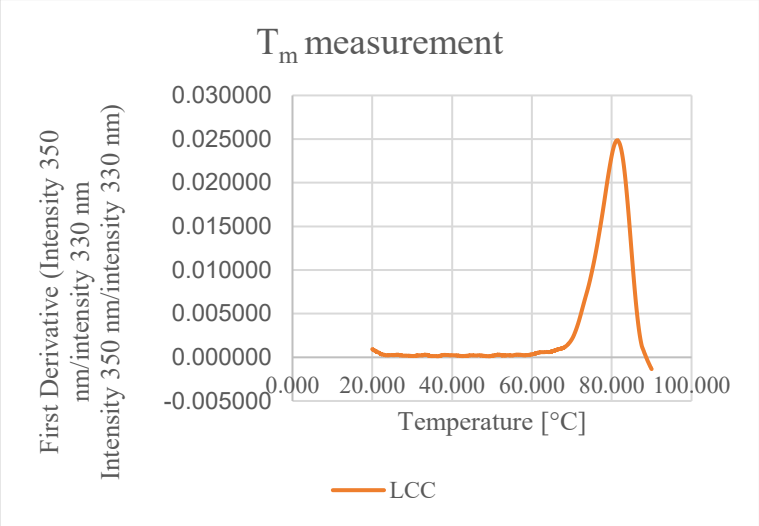


Figure 7. 3 *Tm* measurement of LCC express as first derivative of the ratio between intensity at 350 nm and 330 nm varying the temperature

7.2 Standards analysis

A complete degradation of the polymer would have produced their building blocks, such as dimers and monomers. Since it was not possible running the dimers themselves, it was decided to analyse the HPLC of the monomers dispersed in the potassium phosphate buffer. However, before doing so, all the monomers UV spectra were tested. The results, in figure 7.4, showed a peak at 230 nm, meaning that this wavelength was the optimal one to lead the HPLC investigations.

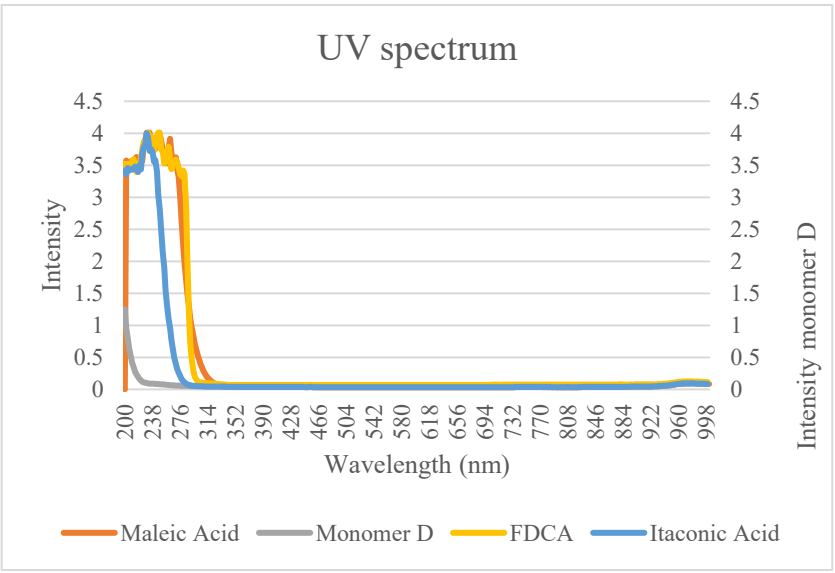


Figure 7. 4 UV intensity vs wavelength (nm) of the monomers

The buffer itself was tested with the HPLC, chromatogram in figure 7.5. The UV detector showed a peak at 0.298 min, with a molecular weight of 191 g/mol in the MS detection.

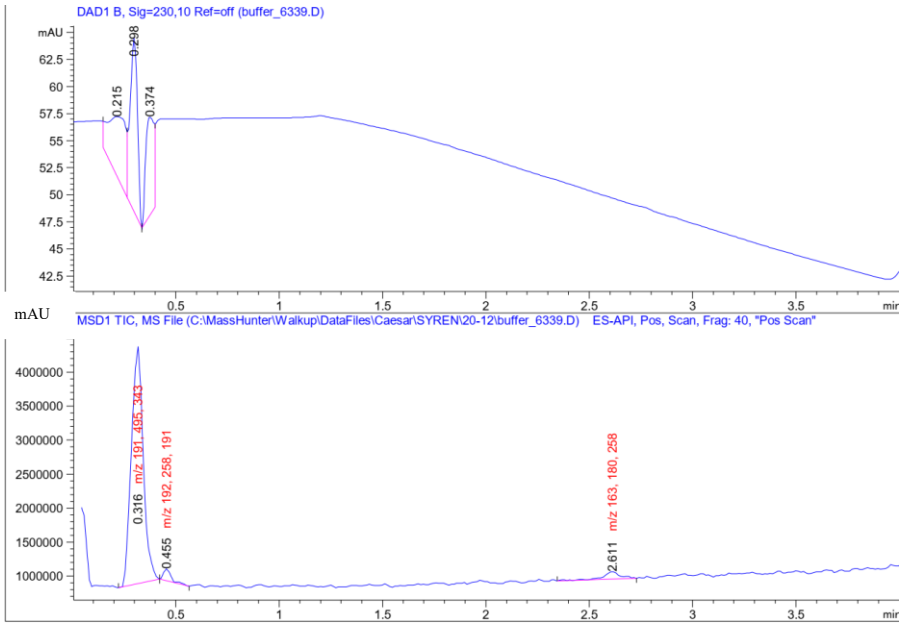


Figure 7. 5 HPLC chromatogram of potassium phosphate buffer, UV detector 230 nm (top) and MS detector (bottom)

Considering the HPLC results for all the monomers, the buffer peak will not be analysed.

For monomer D, the analysis was disturbed with other components, but its characterising peak was in the UV spectrum:

- 0.447 min, with a molecular weight of 147 g/mol.

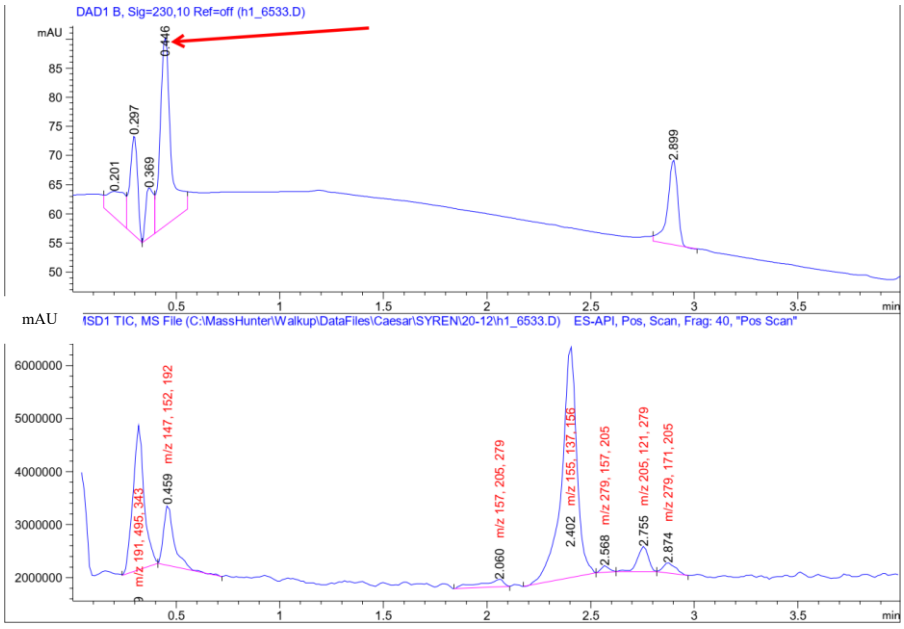


Figure 7. 6 HPLC chromatogram of 0.05 mg monomer D dissolved in potassium phosphate buffer, UV detector 230 nm (top) and MS detector (bottom)

Having performed HPLC with different ratios between solid and liquid part, visible in the appendix table 10.1, it was possible to obtain the calibration curve, in figure 7.7.

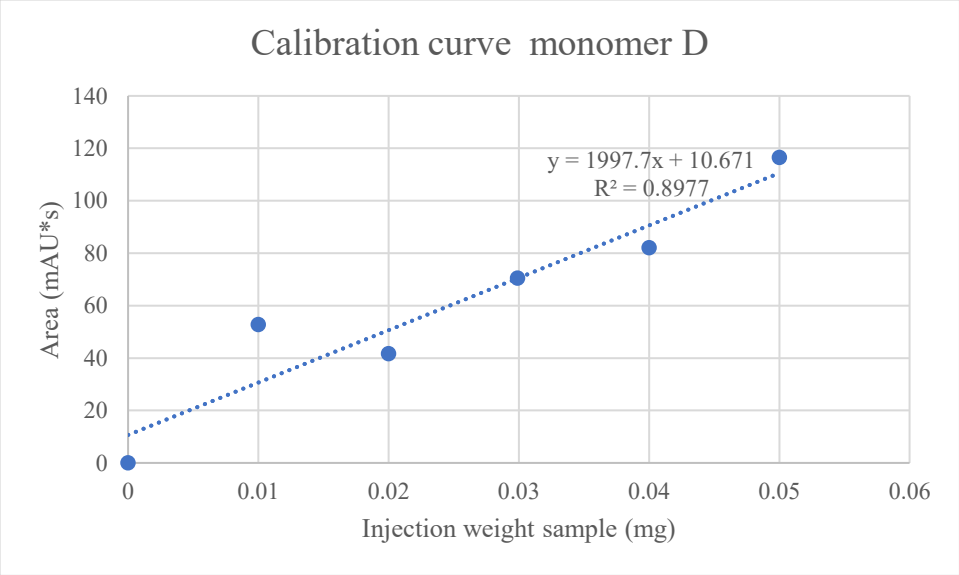


Figure 7. 7 Calibration curve monomer D

For maleic acid, monomer A, the peak was at 0.377 min, with a molecular weight of 117 g/mol. In this case the MS analysis was correct, since maleic acid has a molecular weight of 116 g/mol.

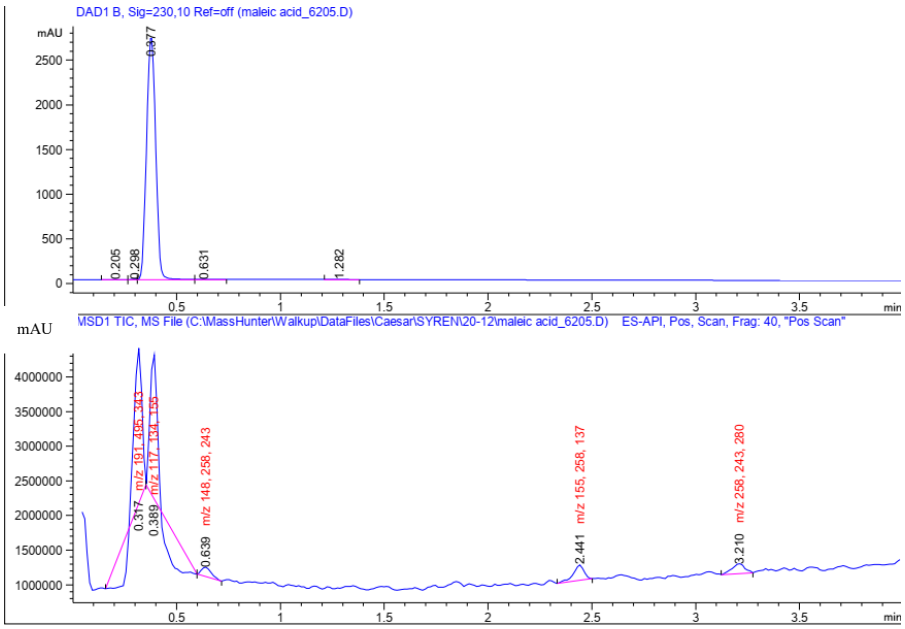


Figure 7. 8 HPLC chromatogram of 0.02 mg Monomer A dissolved in potassium phosphate buffer, UV detector 230 nm (top) and MS detector (bottom)

However, the calibration curve of this compound was not precise, with a R^2 value of just 0.7433, as it can be seen in figure 7.9. This was due to very similar response, in terms of area, even with different samples weight injected, especially in measure 2 and 3, likely due to saturation of detector.

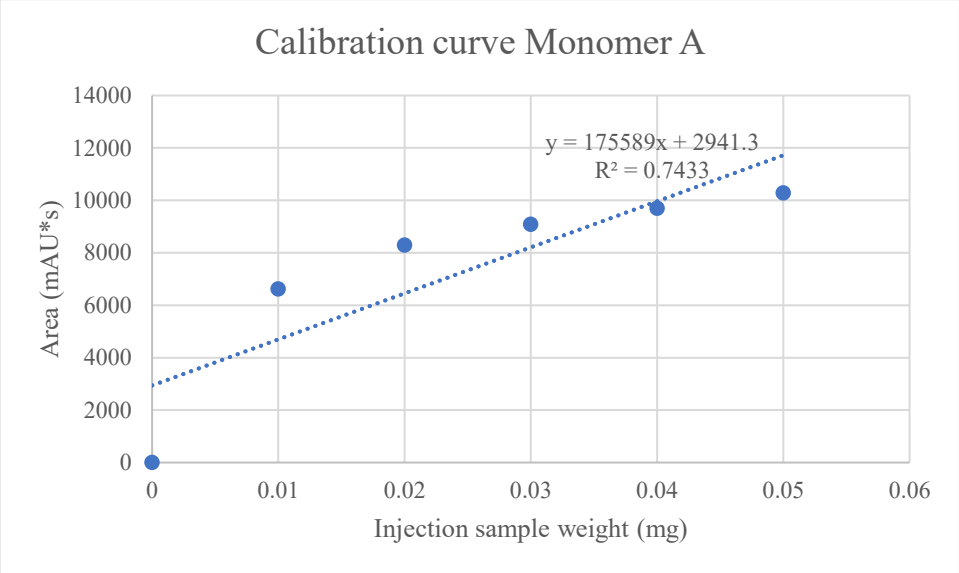


Figure 7. 9 Calibration curve Monomer A

Itaconic acid, monomer B, had a peak of 131 g/mol (the real molecular weight is 130 g/mol) at 0.643 min and a precise calibration curve, figure 7.10 and 7.11.

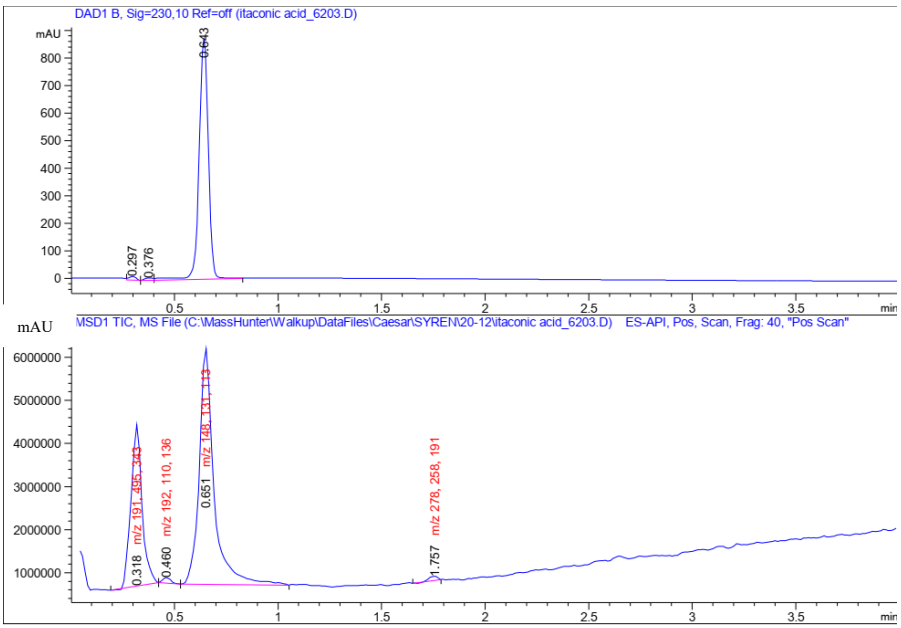


Figure 7. 10 HPLC chromatogram of 0.02 mg Monomer B dissolved in potassium phosphate buffer, UV detector 230 nm (top) and MS detector (bottom)

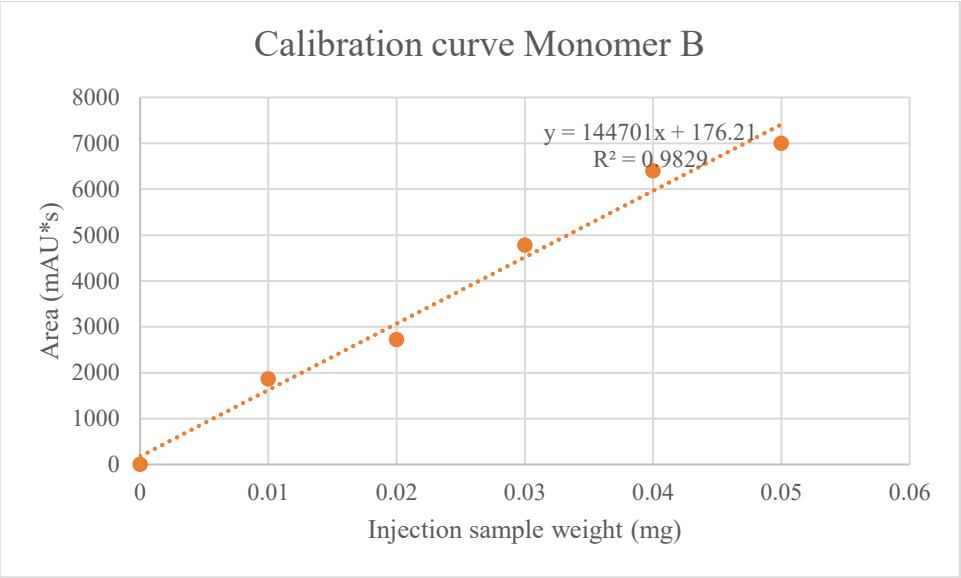


Figure 7. 11 Calibration curve Monomer B

Finally, monomer C, FDCA, was found to be insoluble in most solutions. Despite that, in methanol and buffer it gave the peak at 0.810 min with a molecular weight of 157 g/mol; monomer C molecular weight is 156 g/mol, so the measure was, in this case, accurate, figure 7.12. The calibration curve, figure 7.13, was precise, exception for the fourth measure, when 0.04 mg monomer C were injected.

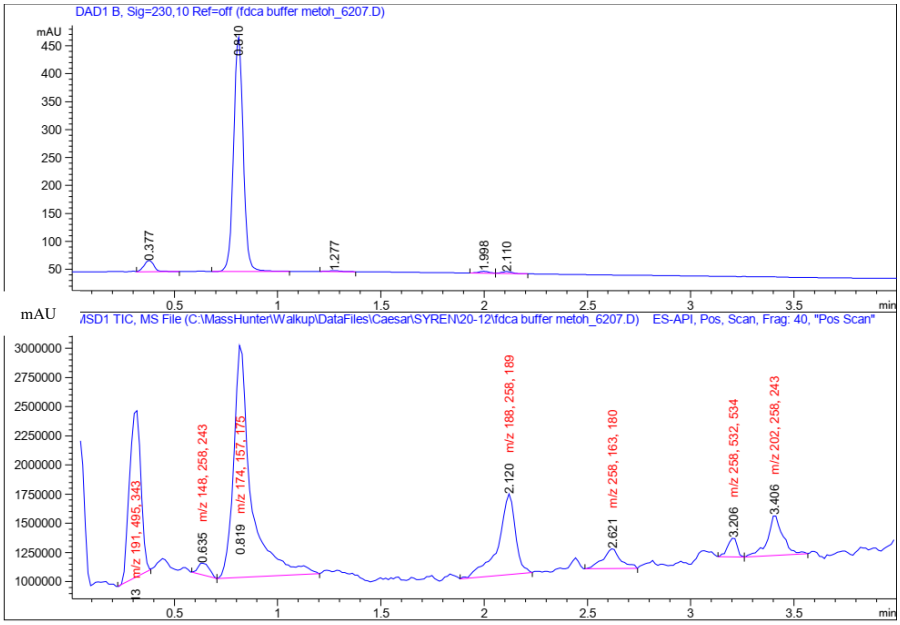


Figure 7. 12 HPLC chromatogram of 0.013 mg monomer C dissolved in potassium phosphate buffer, UV detector 230 nm (top) and MS detector (bottom)

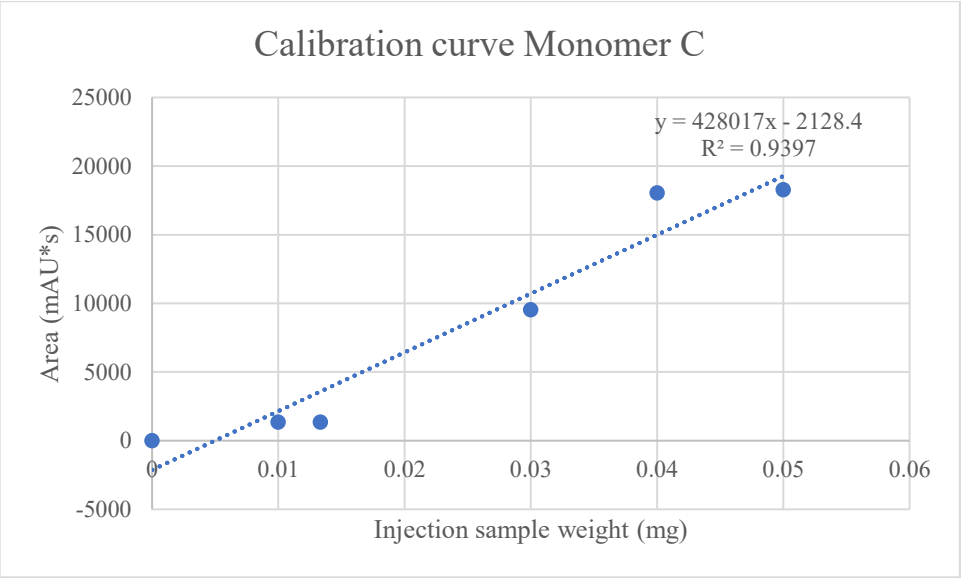


Figure 7. 13 Calibration curve monomer C

7.3 HPLC PETase application analysis

HPLC results for the all the polymers treated with PETase, concentration 0.86 µg/µl, are showed in this paragraph. Disrespecting the concentration, the washing, or the type of polymer, all the product peaks came later than the monomers. This means that the degradation was likely to produce only oligomers, with higher molecular weight and hydrophobicity compared to the monomers, thus, having a higher retention time with a phenyl column. The oligomers production occurred also in PET substrate, as explained in chapter 4, and the degradation was completed using MHETase, able to hydrolase oligomers to monomers.

Looking at P1, all the HPLC results were not showing high quantity of the final products. The control, without enzyme, in figure 7.14 after 48 hours showed two small peaks only in the MS ESI-API detector, at 2.351 min and 3.006 min, while in the UV at 230 nm just the buffer was detected. The molecular weight of those products, just below 300 g/mol, meant that they probably were oligomers, although no enzyme had been used. It is not surprising, considering that hydrolysis is certainly a spontaneous reaction, but it is slow.

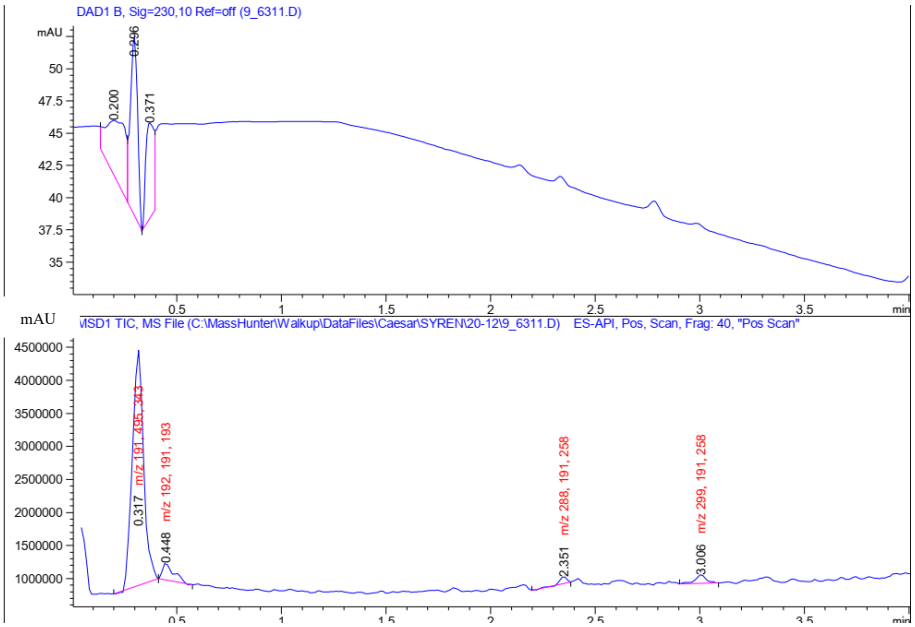


Figure 7. 14 HPLC chromatogram of P1 washed after 48 hours incubation with 0 µg PETase/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

The application of the enzyme should have increased the reaction rate, but the results when a low concentration of PETase (1 µg/5 mg plastic) was used after 48 hours, were not clear, as it can be seen in figure 7.15 especially at the end of the measure. Moreover, the measure had been covered by some background noise. In any case no real product was released. This is explainable considering the preference that PETase has on semi-aromatic polyesters, such as PET, and P1 is an aliphatic polymer.

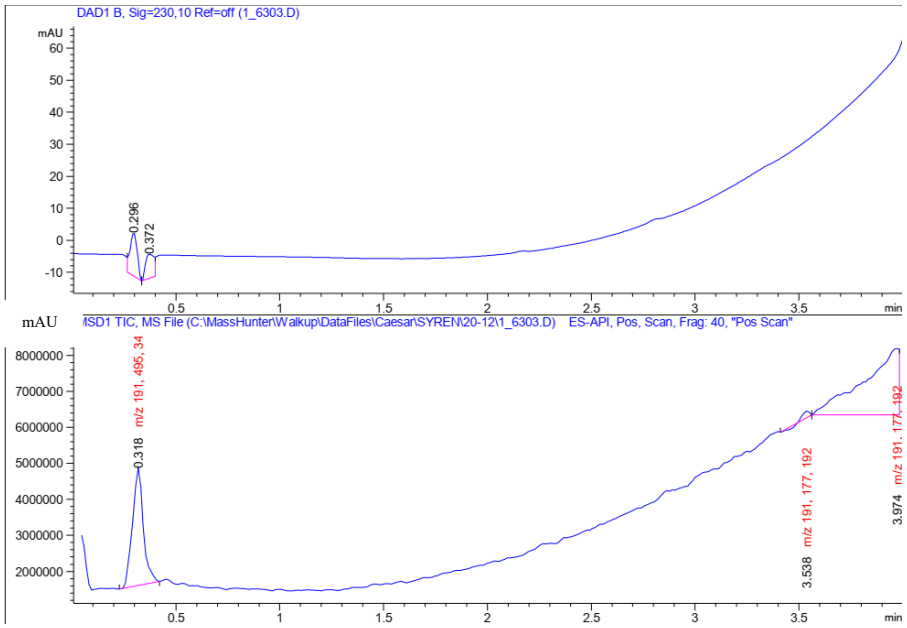


Figure 7. 15 HPLC chromatogram of P1 washed after 48 hours incubation with 1 µg PETase/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

With a concentration of 15 µg PETase/5 mg plastic, on the other hand, some products had been released, figure 7.16. Two peaks at 2.336 min and 2.989 were visible in in the UV spectrum and several oligomers appeared with a molecular weight between 200 g/mol and 312 g/mol. The two peaks had respectively an area of 12.48722 mAU*s and 5.45732 mAU*s. To convert these results in weights, since there was no calibration curve for dimers, one of the monomers will be used: monomer B has the highest R^2 value so it was chosen, after being slightly modified to become an intercept in the axis origin, equations 7.1 and 7.2.

$$y = 149507x \quad (7.1)$$

$$R^2 = 0.9813 \quad (7.2)$$

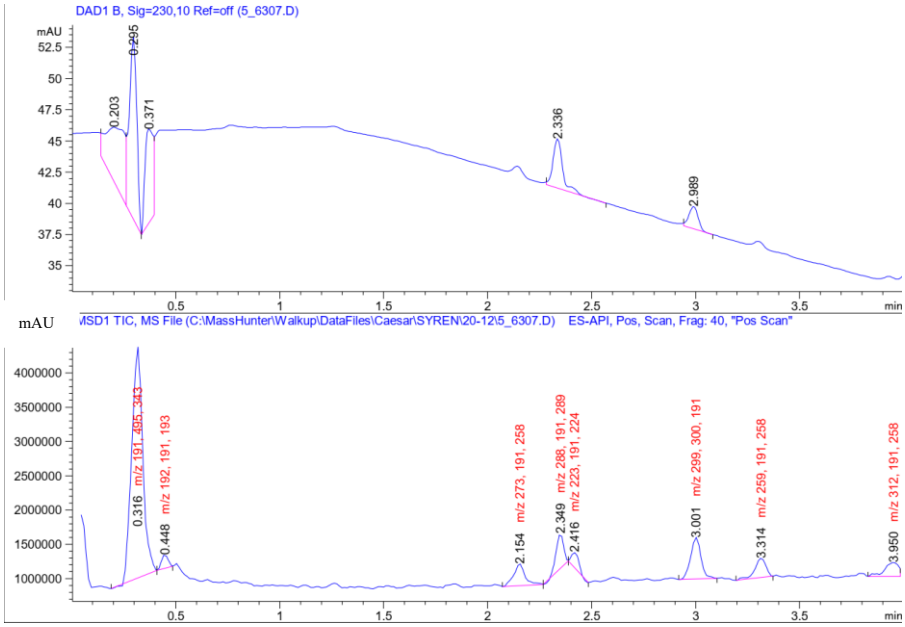


Figure 7. 16 HPLC chromatogram of P1 washed after 48 hours incubation with 15 µg PETase/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

The two resulting products were presented with weights of $8.35226*10^{-5}$ mg and $3.65021*10^{-5}$ mg respectively. From this moment on, the area values will be directly shown as weights and all the products weights and results, as well as retention times, can be seen in the appendix.

The other possibility was to have the degradation without the previous washing. Although the control, figure 7.17, did not show appreciable differences with the washed one, this was not true if the enzyme was used, figure 7.18.

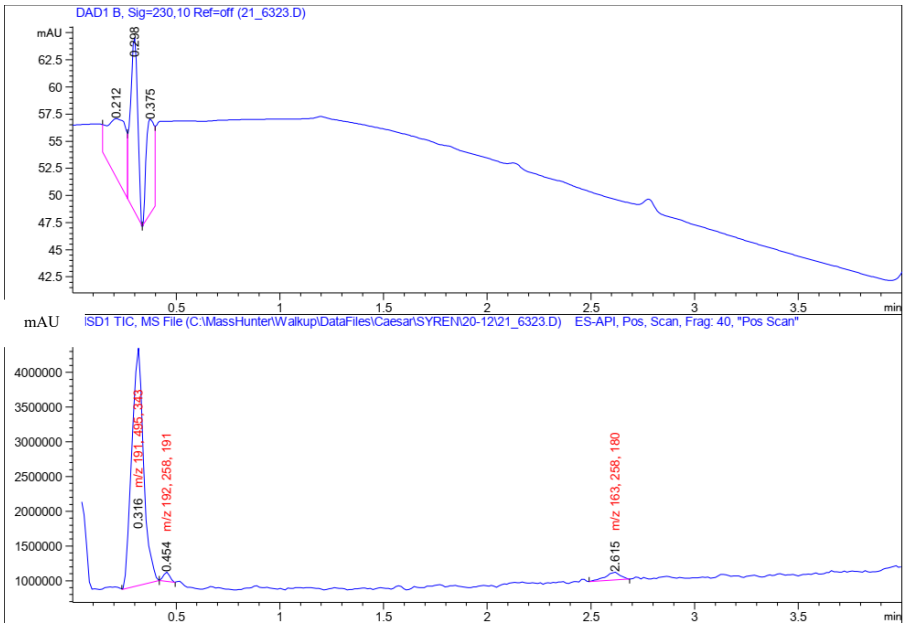


Figure 7. 17 HPLC chromatogram of P1 non washed after 48 hours incubation with 0 µg PETase/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

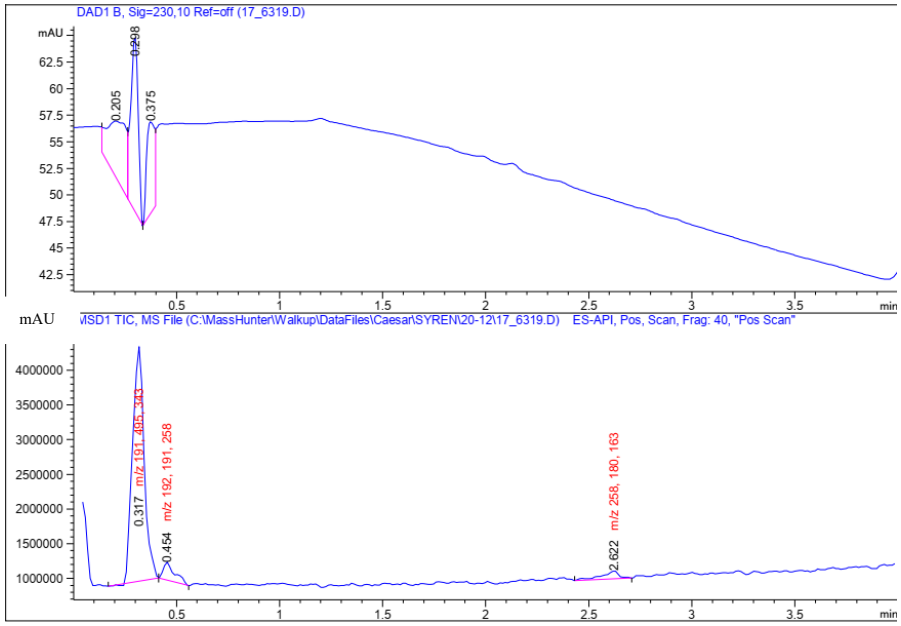


Figure 7. 18 HPLC chromatogram of P1 non washed after 48 hours incubation with 15 µg PETase/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

In this case no results were found: the washing treatment was essential for the enzyme to enter the substrate surface, without any other protein or charge competing with it. Therefore, after a first trial with both possibilities, it was decided to test only washed samples.

P2 is the other aliphatic polymer and neither in this case the degradation was intense. The samples without enzyme showed some differences between 24 and 48 hours, respectively figure 7.19 and 7.20, confirming that these degradation products were formed during the incubation, even without a catalyst.

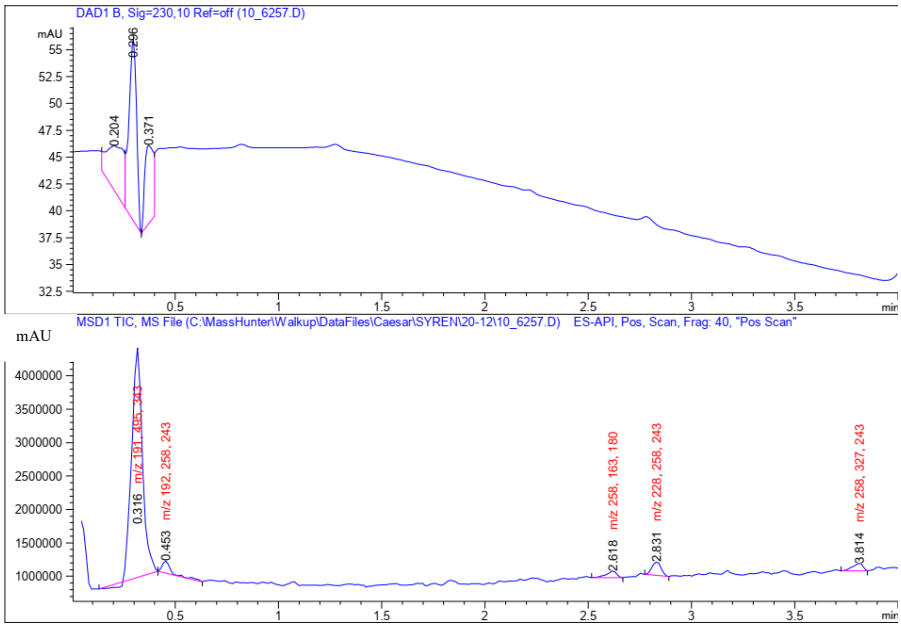


Figure 7. 19 HPLC chromatogram of P2 washed after 24 hours incubation with 0 µg PETase/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

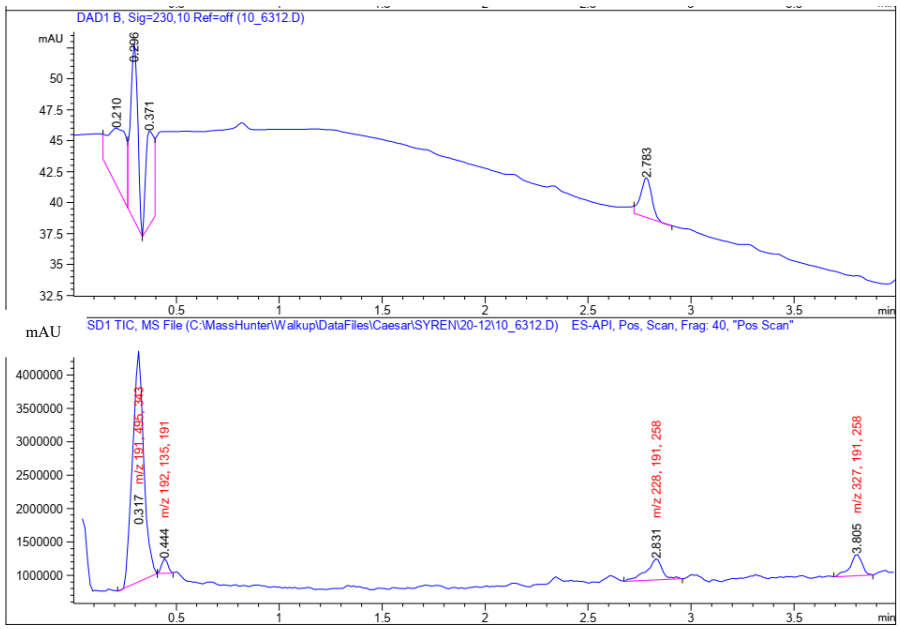


Figure 7. 20 HPLC chromatogram of P2 washed after 48 hours incubation with 0 µg PETase/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

When 1µg PETase per 5 mg plastic was used, the degradation velocity increased, as figure 7.21 shows. If the 48-hours control was compared with the 48-hours low concentration of enzyme in the 230 nm UV spectrum, a peak at 2.344 min emerged. The enzyme was probably able to cut the polymer in precise regions, in some other way than the simple hydrolysis without catalyst.

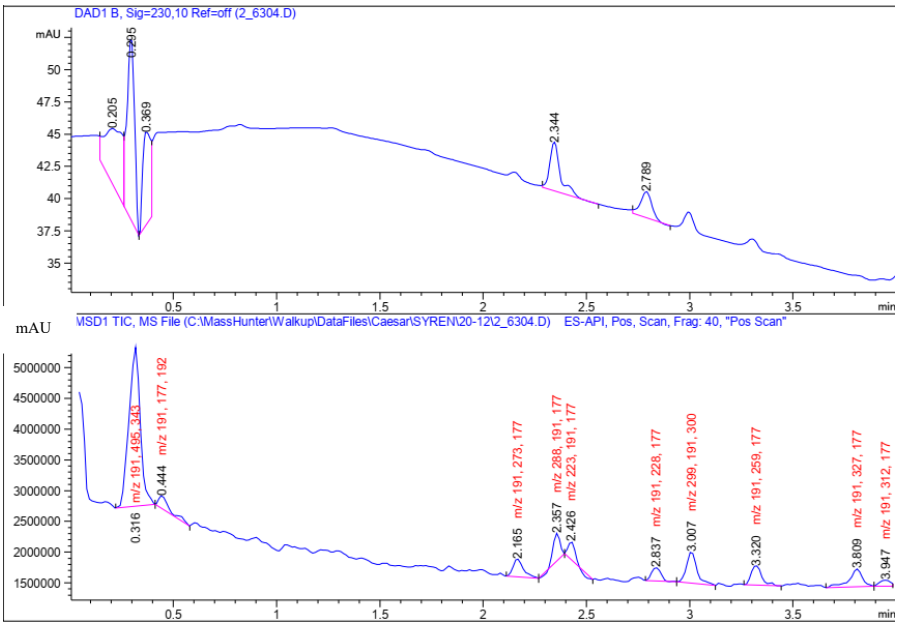


Figure 7. 21 HPLC chromatogram of P2 washed after 48 hours incubation with 1 µg PETase/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

The high concentration of PETase, as for P1, did not giving any results in degrading P2, showing, in figure 7.22, that a high content of the enzyme had the only effect of increasing the competitivity of the proteins.

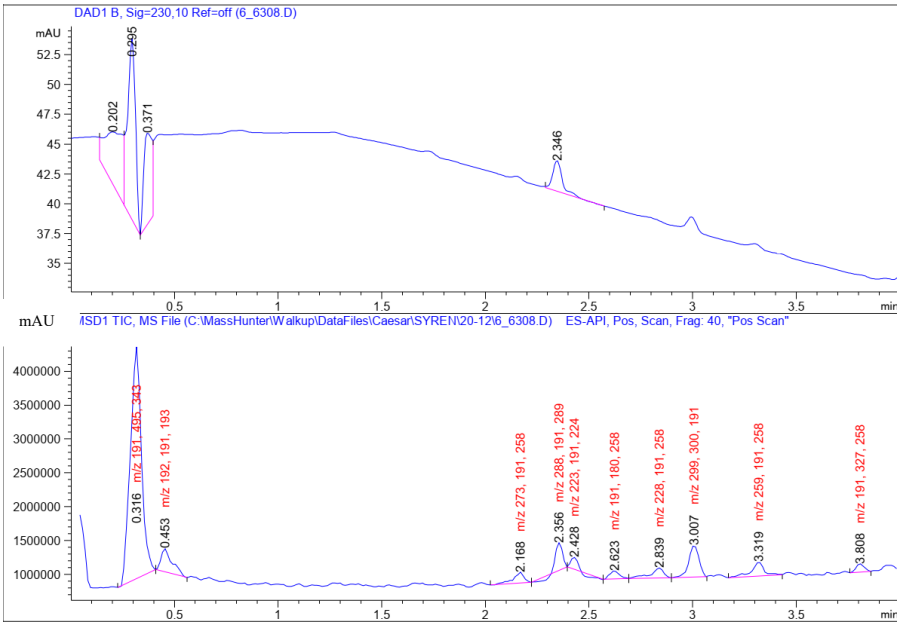


Figure 7. 22 HPLC chromatogram of P2 washed after 48 hours incubation with 15 µg PETase/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

P3 is a semi-aromatic copolymer; but although its structure is one of the most complicated among the studied polymers, the HPLC on its degradation products showed the best results. The sample without enzyme, figure 7.23, showed no peaks at all, meaning that in this case

the spontaneous hydrolysis was even slower, probably due to the presence of four different monomers alternating.

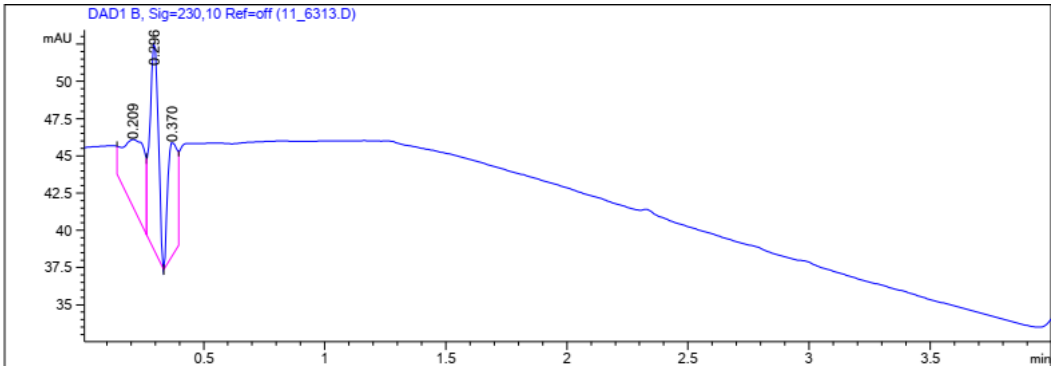


Figure 7. 23 HPLC chromatogram of P3 washed after 48 hours incubation with 0 µg PETase/5 mg polymer, UV detector 230 nm

On the other hand, with a low concentration of PETase, three high peaks appeared in the chromatogram, figure 7.24. Their retention times, 2.344 min, 2.997 min and 3.306 min confirmed the hydrophobicity and the high mass of the products and, at least for the first two, a resemblance with the degradation products of P1 and P2 can be seen. This meant that they could have been the same kind of oligomer. In this case, however, the quantity of these oligomers, $2.453*10^{-4}$ mg, $1.192*10^{-4}$ mg and $3.951*10^{-5}$ mg, suggested that the hydrolysis was faster and more effective: PETase was able to degrade better substrates with a benzene ring, due to the conformation of its active site, as it had been explained in chapter 4.

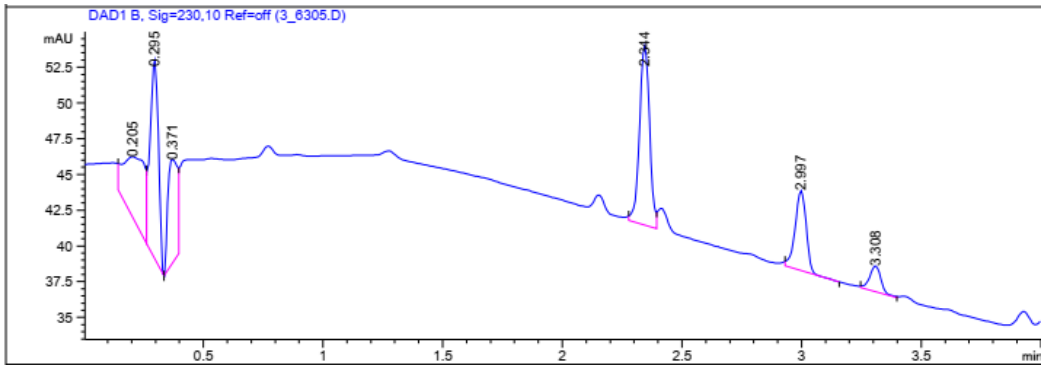


Figure 7. 24 HPLC chromatogram of P3 washed after 48 hours incubation with 1 µg PETase/5 mg polymer, UV detector 230 nm

When the procedure to characterize the released products was chosen, it was not known whether these would have been oligomers or monomers, so, considering the low solubility of FDCA, monomer C, in the simple buffer, it was decided to run another HPLC with methanol. However, the results showed in figure 7.25, not only confirmed that the monomer, with a retention time of 0.859 min, did not appear, but also covered the oligomers peaks. That considered, it had been decided not to show the chromatograms with methanol.

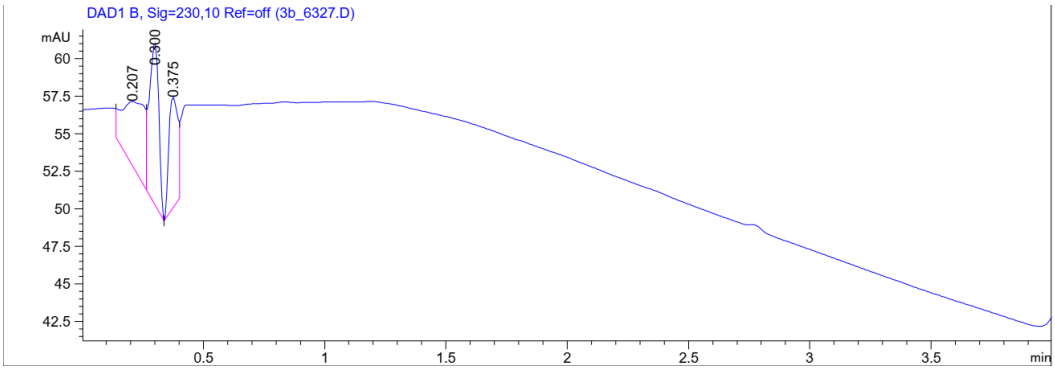


Figure 7. 25 HPLC chromatogram of P3 washed after 48 hours incubation with 1 µg PETase/5 mg polymer and mixed with methanol, UV detector 230 nm

Finally, when a high concentration of PETase was applied on the sample, no major peaks were visible, figure 7.26, confirming that the quantity of the enzyme was not increasing the speed, but it was even able to decrease it, with just few active sites being really occupied by the substrate.

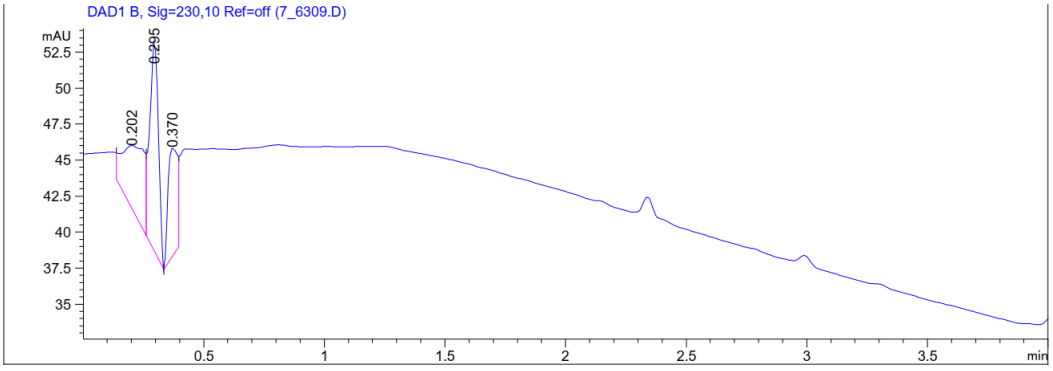


Figure 7. 26 HPLC chromatogram of P3 washed after 48 hours incubation with 15 µg PETase/5 mg polymer, UV detector 230 nm

Since P3 results were the clearest, it is interesting to focus more on the molecular weight of the obtained oligomers. P3 is composed by two dimers: D1, figure 7.27 molecular weight 272 g/mol, and D2, figure 7.28 molecular weight 310 g/mol.

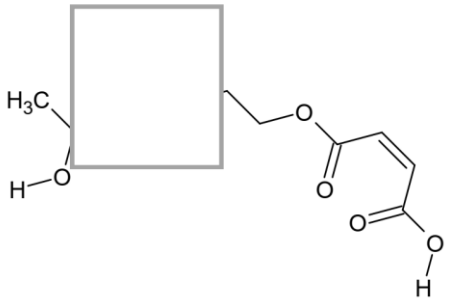


Figure 7. 27 Dimer 1, D1, of P3

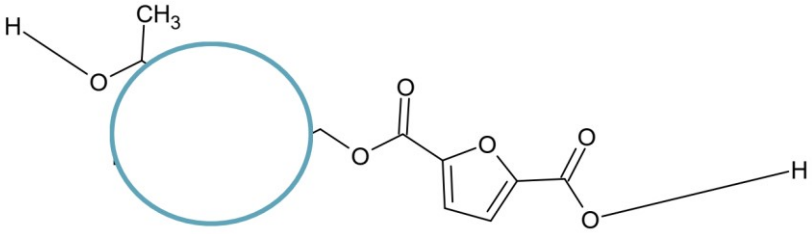


Figure 7. 28 Dimer 2, D2, of P3 and P4

Although all the polymers were in their crosslinked form, the enzyme could have hydrolysed only the ester bond; however, this might have produced some differences in the weight of the products. This said, when looking at the MS chromatograms of the 48 hours HPLC of these polymers, some results may be now understood.

Figure 7.29 represents MS HPLC of P3; among all the peaks, excluding the first four, part of the buffer, the weight was always between 260 g/mol and 313 g/mol. So, it can be said that the products of this degradation were the dimers, which also exited later compared to the monomers with retention times between 0.300 min and 0.900 min.

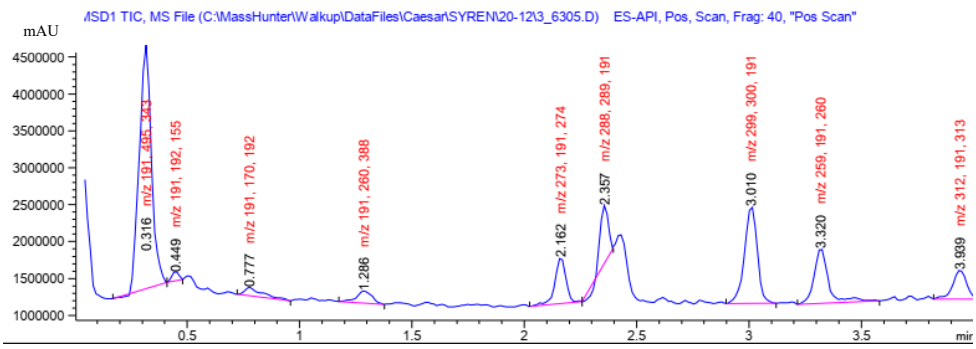


Figure 7. 29 HPLC chromatogram of P3 washed after 48 hours incubation with 1 µg PETase/5 mg polymer MS detector

The same observation can be done also for P4, which dimers are D2 and D3, with a molecular weight of 312 g/mol, figure 7.30.

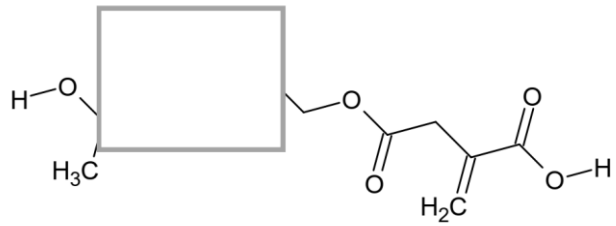


Figure 7. 30 Dimer3, D3, of P4

The HPLC results for P4 resembled P3 ones: as the control, figure 7.31 confirms that nothing was produced in the non-catalysed hydrolysis, the low concentration of PETase formed two distinct oligomers, with retention times of 2.338 min, $1.608*10^{-4}$ mg, and 2.990 min $7.761*10^{-5}$ mg, figure 7.32.

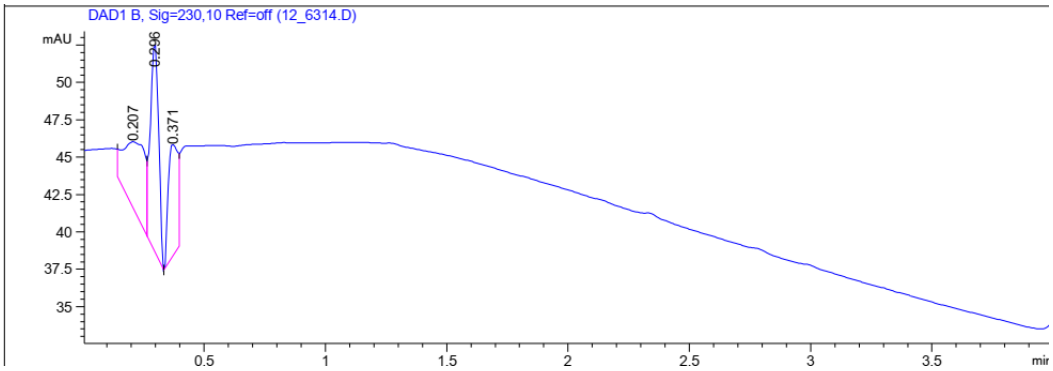


Figure 7. 31 HPLC chromatogram of P4 washed after 48 hours incubation with 0 µg PETase/5 mg polymer, UV detector 230 nm

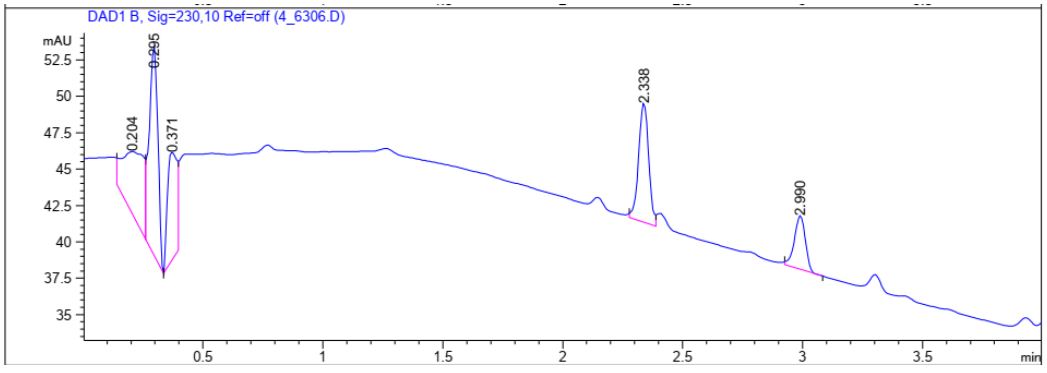


Figure 7. 32 HPLC chromatogram of P4 washed after 48 hours incubation with 1 µg PETase/5 mg polymer, UV detector 230 nm

With a high concentration of PETase, figure 7.33, nothing was visible, supporting the thesis of the enzyme competitivity towards the substrate.

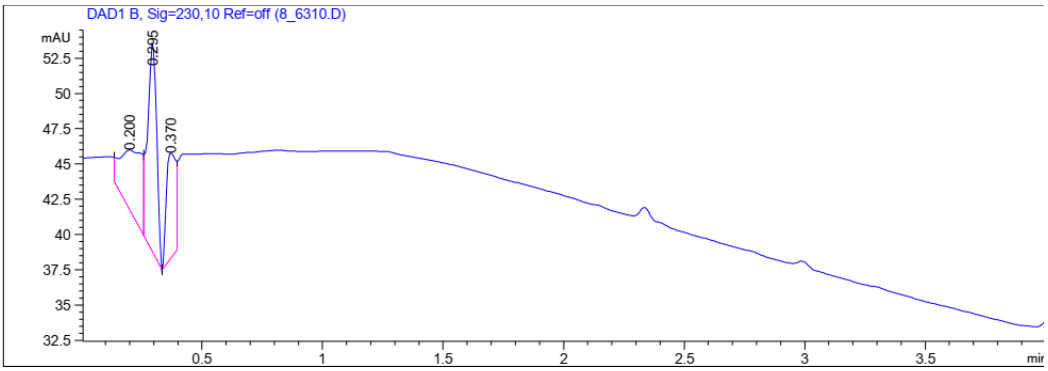


Figure 7. 33 HPLC chromatogram of P4 washed after 48 hours incubation with 15 µg PETase/5 mg polymer, UV detector 230 nm

Considering the MS detector results for the low concentrations, in figure 7.34, less clear than P3, the molecular weight was between 260 g/mol and 313 g/mol. It was still possible that they represented dimers.

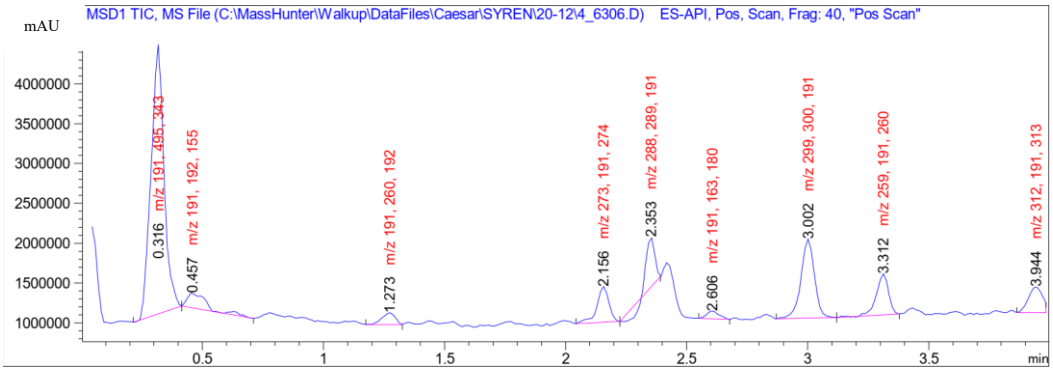


Figure 7. 34 HPLC chromatogram of P4 washed after 48 hours incubation with 1 µg PETase/5 mg polymer, MS detector

Although these results are important, considering that for the monomers some molecular weight shifting was visible in the HPLC MS, it was decided to use also the GC for P1 and P2 when the lowest PETase concentration was applied, the best results obtained.

Finally, comparing the results of P3 and P4 degradation, similar dimers appeared around 2.300 min and 2.990 min after 48h incubation, with higher quantity in both cases for the first polymer, showing a better selectivity of the enzyme towards monomer A, figure 7.35.

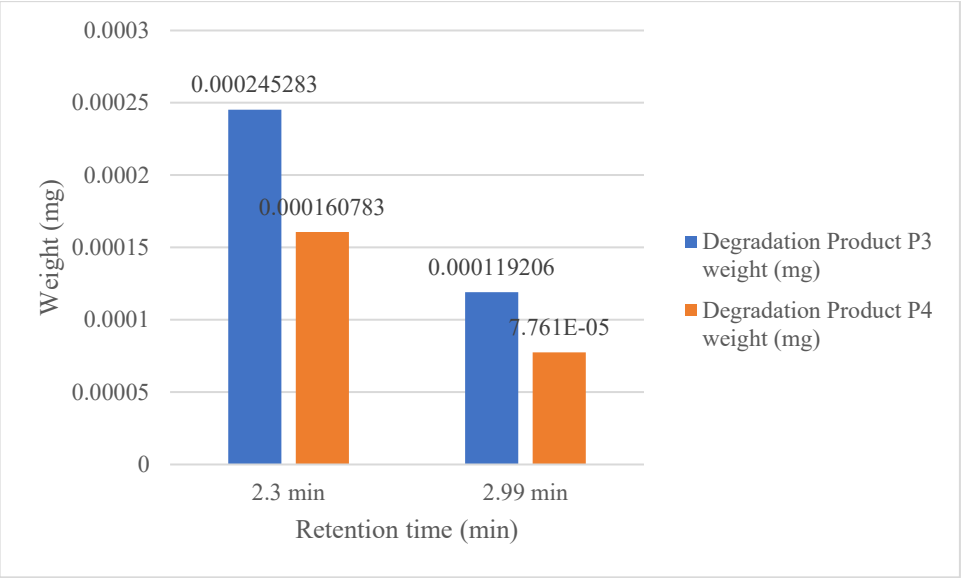


Figure 7. 35 Chart comparing the quantity of similar retention time degradation products for P3 and P4 after 48h

7.4 GC PETase application analysis

GC results for polymer P3 and P4 both confirmed what it had been observed through MS of HPLC. In figure 7.36 there is the gas chromatogram of P3.

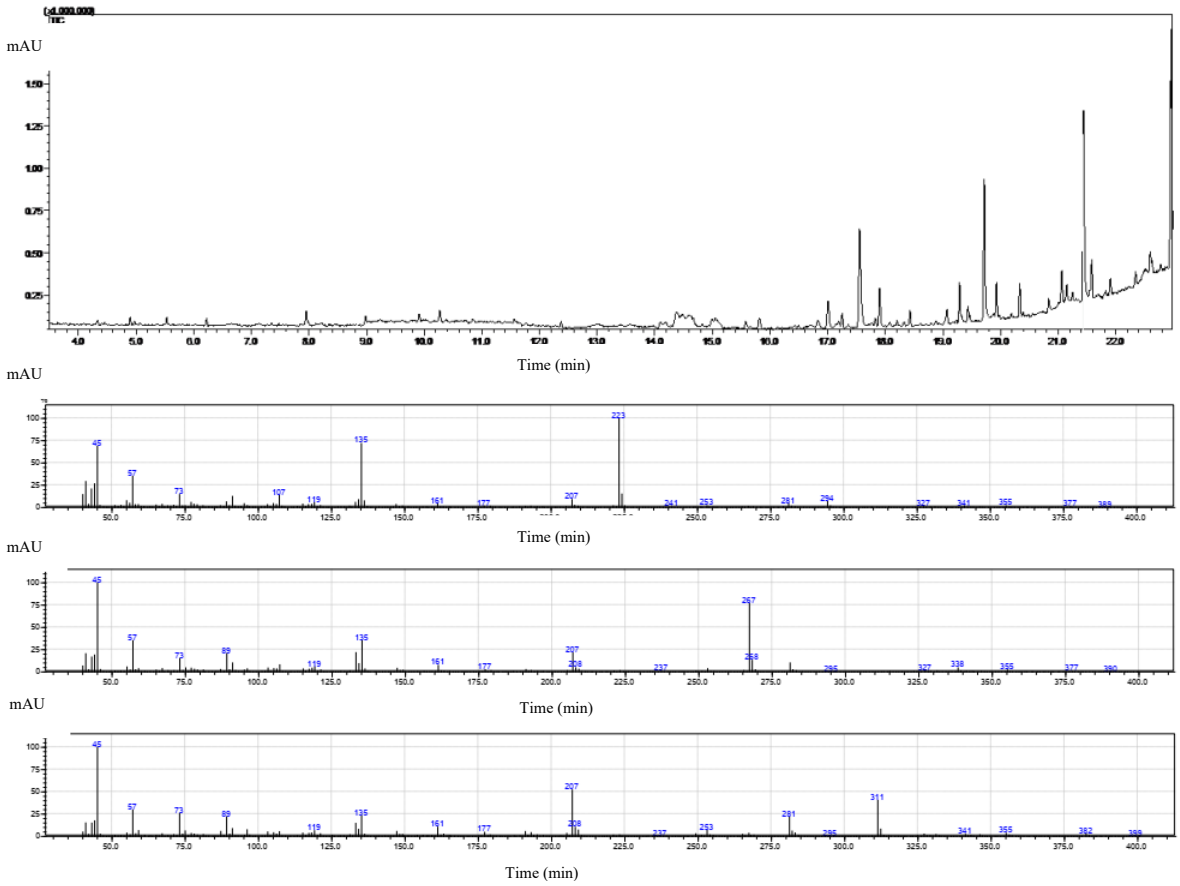


Figure 7. 36 GC chromatogram of P3 washed after 48 hours incubation with 1 µg PETase/5 mg polymer, MS detector

Three peaks were visible after 17 minutes: they were composed by many oligomers, as the zoom shows, but the two most important were 311 g/mol and 267 g/mol, which might represent the oligomers. There were also other peaks at 223, 207, 135 and 45 g/mol; even though this last one seemed to be part of the buffer, it could have been a salt for example, the others might still represent oligomers, which were cut in different positions compared to the canonical dimers.

The GC results for the products released by P4 hydrolysis were less clear compared to the P3 ones, but confirmed that some oligomers, with a molecular weight between 207 g/mol and 281 g/mol were formed, as it can be seen from figure 7.37.

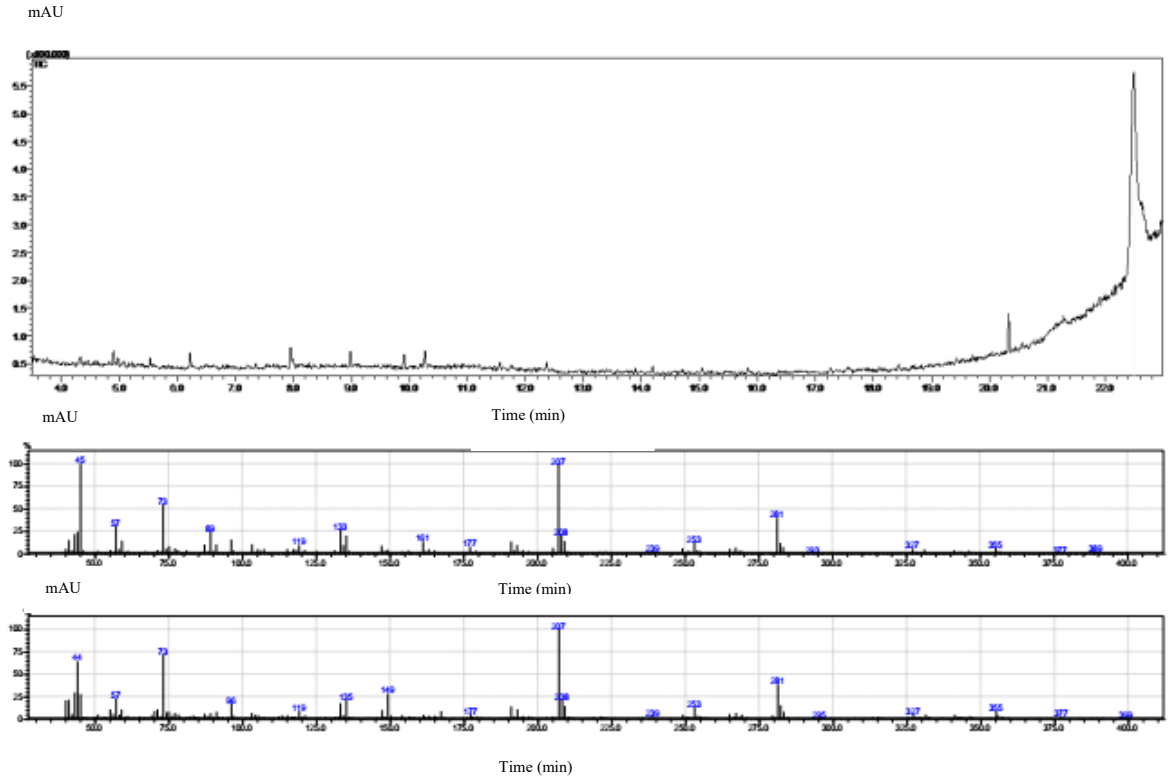


Figure 7. 37 GC chromatogram of P4 washed after 48 hours incubation with 1 µg PETase/5 mg polymer, MS detector

7.5 SEM PETase application analysis

As it was explained in chapter 3.2.2, the place of hydrolysis depends on many parameters and it can be on the surface or in the bulk: SEM analysis was performed in order to obtain proof that in this case the reaction speed was higher than the degradation one. Unfortunately, SEM results were the most unclear of all and they did not show any kind of correlation with the HPLC and GC results. This technique was performed on all the samples showing better results for the HPLC, so only the washed ones, and their controls, to check whether the degradation was occurring on the surface, forming holes or other structures.

In figure 7.38 is visible P1 treated with 15 µg/5 mg plastic.

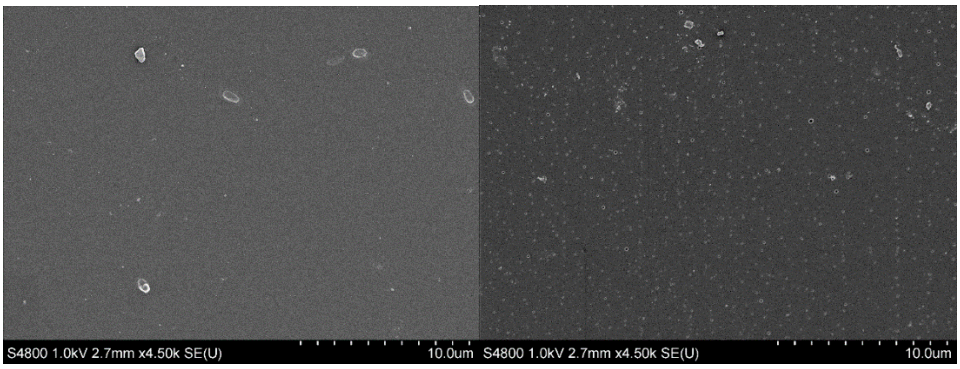


Figure 7. 38 SEM images for P1, 0 µgPETase/5 mg polymer (left) and 1 µgPETase/5 mg polymer (right), both washed and after 48 hours

There were no holes inside. This probably meant that the degradation happened in some other way rather than forming holes. Moreover, if the control of P1 is analysed, on the left, some small holes, the black circle countered in white, can be seen. This probably meant that the washing or the degradation buffer created them.

This is also true for polymer P2; with the lowest enzyme concentration, figure 7.39 right, and its control, figure 7.39 left. In this last one the bubbles were probably droplets coming from the dried liquid.

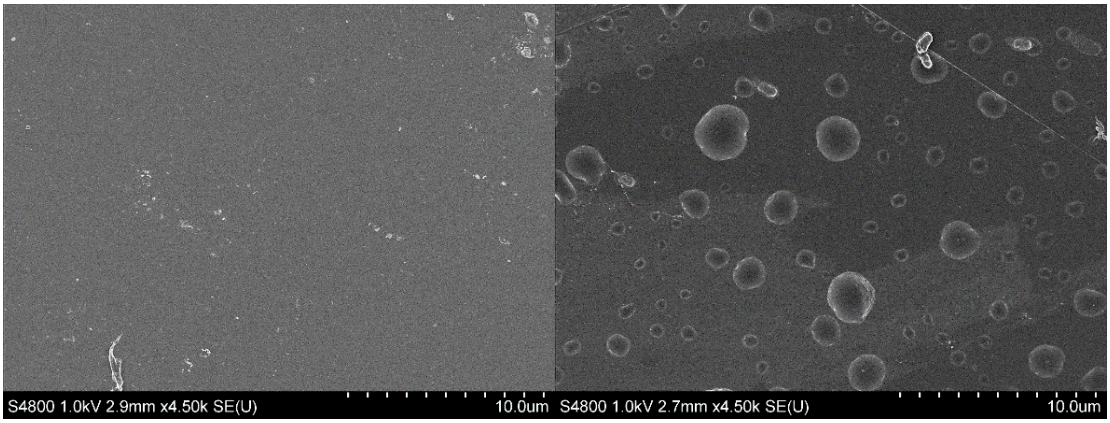


Figure 7. 39 SEM images for P2, 0 µgPETase/5 mg polymer (left) and 1 µgPETase/5 mg polymer (right), both washed and after 48 hours

However, the enzyme could, in some case, especially towards the most suitable polymers, such as P3 and P4, enhance the formation of holes, as it can be seen in low concentration figure 7.40 top right and control 7.40 top left, of polymer P3 and in figure 7.40 bottom right and 7.40 bottom left, low concentration and control of P4.

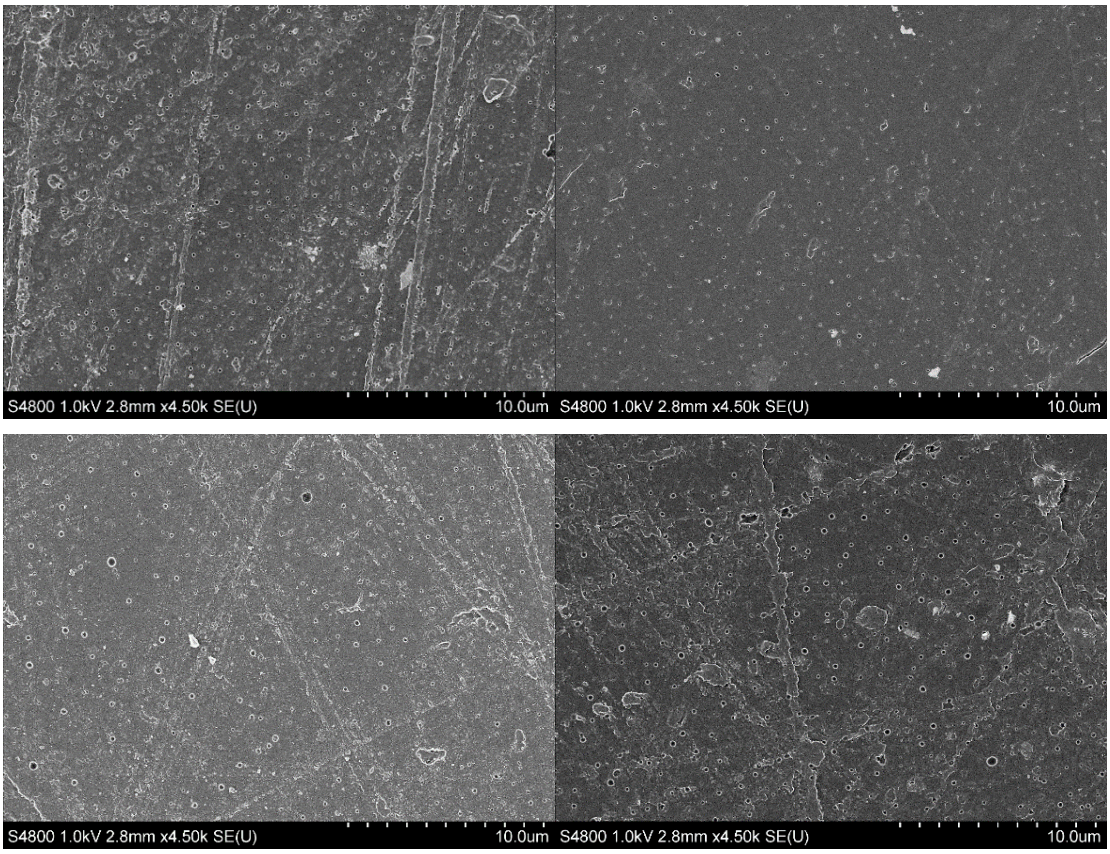


Figure 7. 40 SEM images for P3 (top) and P4 (bottom), 0 µgPETase/5 mg polymer (left) and 1 µgPETase/5 mg polymer (right), both washed and after 48 hours

Despite these considerations, the results were not promising, so it was decided that for the LCC test no SEM measure would have been done.

7.6 HPLC LCC application analysis

LCC concentration at the end of the purification step was 20 µg/µl, so, before the application, it was diluted with Tris 10 mM until it reached a value of 0.80 µg/µl .The application of LCC gave not only peaks at high retention times, but also some between 0.400 min and 0.800 min. This meant that the degradation was not stopped at the dimer formation, but, in some way, proceed until the monomers were found.

This behaviour, however, was probably enhanced by the high temperature application, since it is visible also in the control samples, where LCC was not used. In the degradation of P1, the control, figure 7.41, there was a small peak at 0.437 min, which could have been monomer D (retention time 0.447 min), although in the MS detection the molecular weight was not 147 g/mol. There were also other peaks after 1.800 minutes, which could have been dimers. The degradation in this case occurred thanks to a thermal hydrolysis: temperature played a role in the simple hydrolysis of PLA, equation 3.17, so it was possible that it did the same in this case, probably enhanced by the shaking of the incubator at 600 rpm.

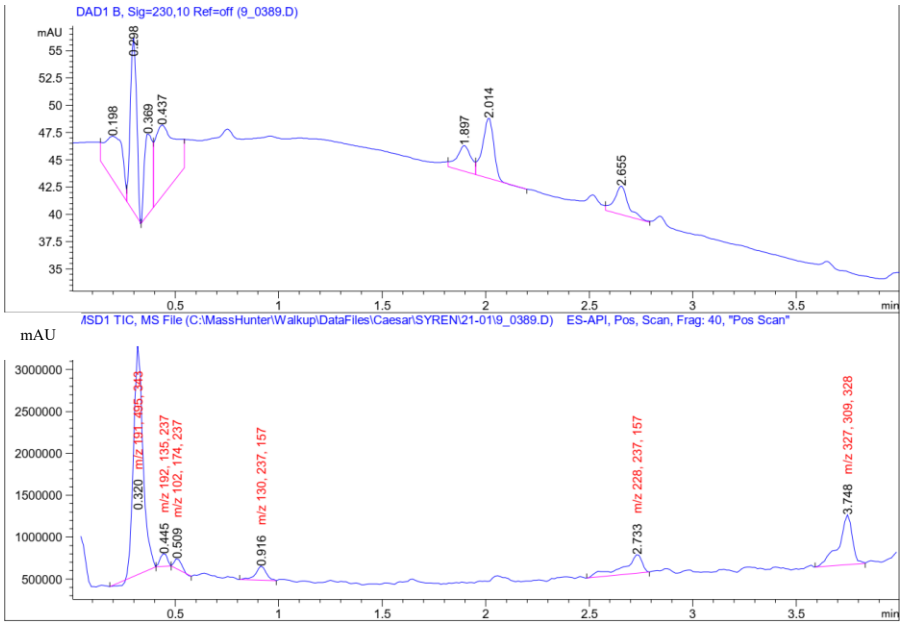


Figure 7. 41 HPLC chromatogram of P1 washed after 48 hours incubation with 0 µg LCC/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

Thermal hydrolysis also helped the enzymatic degradation when 1 µg LCC/5 mg polymer were applied, figure 7.42. There, the peak at 0.441 min, showed a production of a compound with molecular weight that could have been also 147 g/mol. It was even more possible that it was monomer D, with a high production rate. For this reason, the intercept calibration curve of that standard (y=2288.9x) was used to evaluate all the peaks with similar retention time. In this case $4.37*10^{-2}$ mg was formed. Moreover, other peaks after 2 minutes were present in the same composition as before, but with a new one compared to the control (3.053 min and a weight of $3.53*10^{-5}$ mg) appeared.

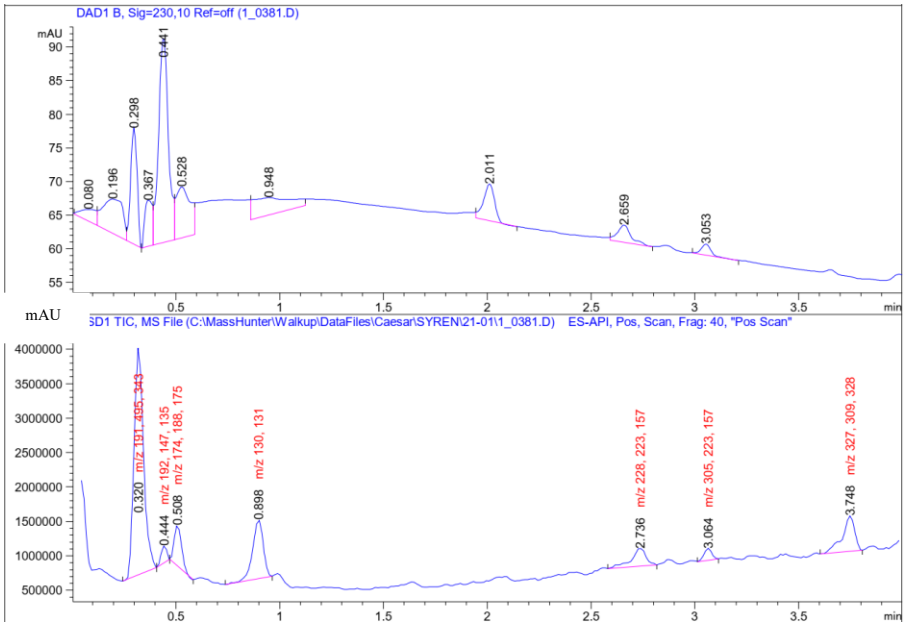


Figure 7. 42 HPLC chromatogram of P1 washed after 48 hours incubation with 1 µg LCC/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

Application of a higher LCC concentration, 15 µg/5 mg polymer, showed a similar behaviour as a high content PETase application did: the characterising monomer peak was lower than before, $2.90*10^{-2}$ mg 0.443 min, even though higher than the control. The interesting result in this case was the high formation of possible dimers at 2.018 with a weight of $3.36*10^{-4}$ mg. It seemed that high content LCC helped the thermal degradation to go faster but did not really persuade the enzymatic catalysis until reaching the monomer, probably because the high content of proteins competed for the substrate. Hence, in this case the degradation was still mainly thermal.

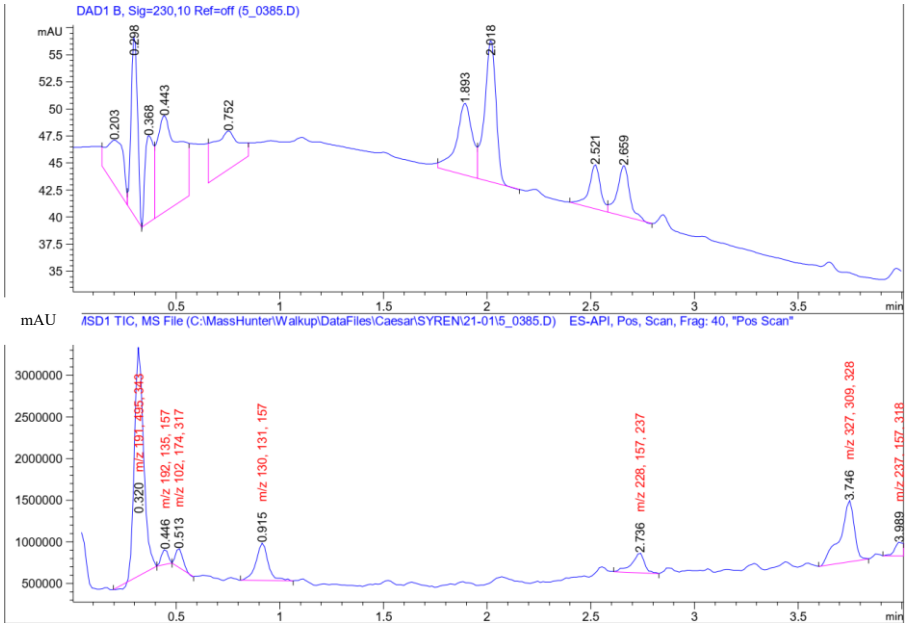


Figure 7. 43 HPLC chromatogram of P1 washed after 48 hours incubation with 15 µg LCC/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

The degradation of P2 resembled the one of P1: there was a thermal degradation enhanced by the low content LCC application, which moved the reaction towards the monomer production, and, partly, by the high concentration of the enzyme, that continued the behaviour of the control.

When analysing the control, figure 7.44, the main difference with P1 is the quantity of the compound exiting at 1.909 min, $4.16*10^{-4}$ mg, although there was no signal at that time in the MS detector.

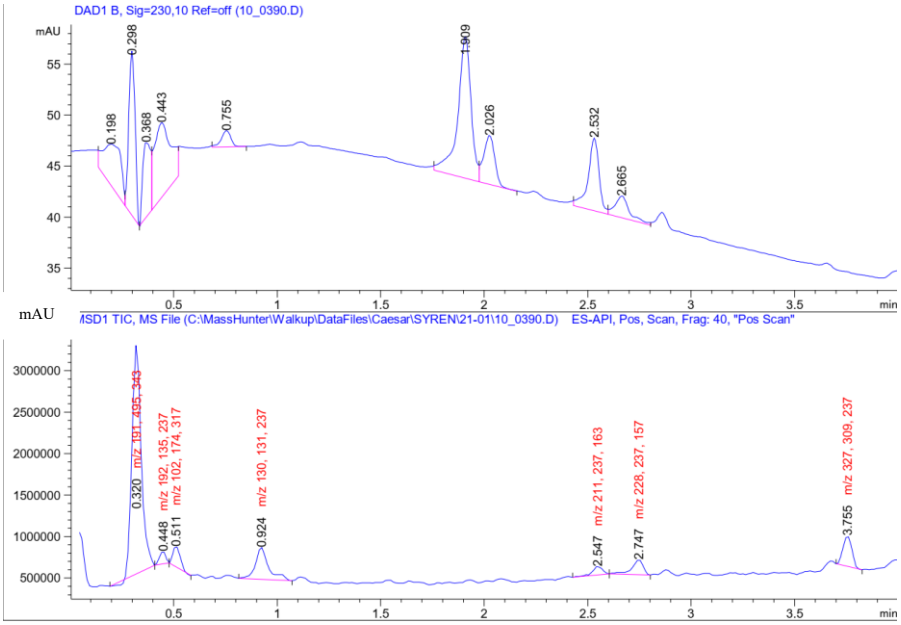


Figure 7. 44 HPLC chromatogram of P2 washed after 48 hours incubation with 0 µg LCC/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

LCC low application produced the same monomer as for P1, it exited at 0.444 min with a weight of $3.82*10^{-2}$ mg and a molecular weight of 147 g/mol. Despite the smaller content compared to P1, monomer D was still one of the degradation products, with other dimers having a retention time higher than 2 min. The other monomer of P2, B, was not analysed in the HPLC chromatogram, even though its presence could have been expected; it was probably hidden in the "dimers" region, united to some of the crosslinks.

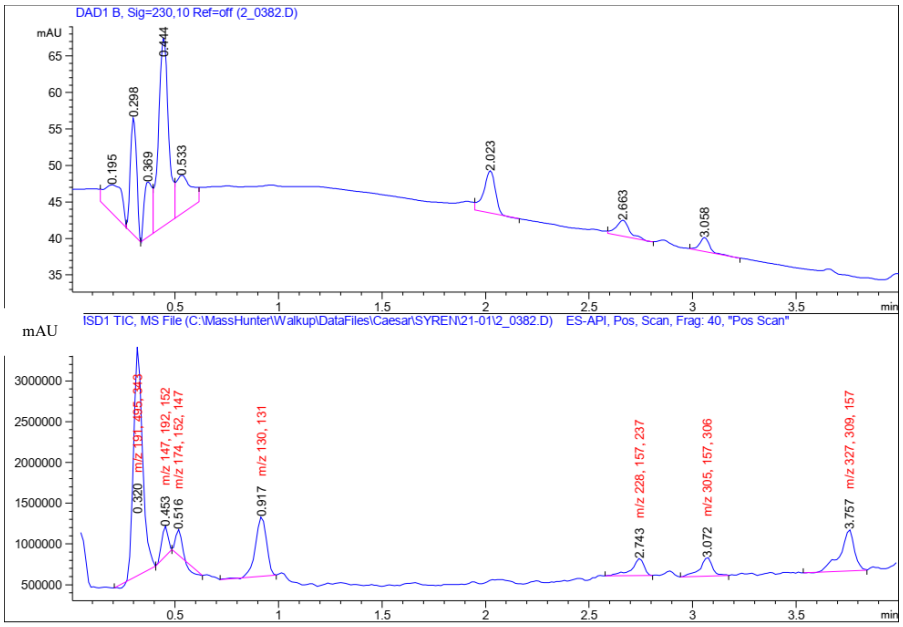


Figure 7. 45 HPLC chromatogram of P2 washed after 48 hours incubation with 1 µg LCC/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

Finally, as said before, figure 7.46 confirmed that high content of LCC enhanced the thermal degradation, with the same peaks as the control: the enzymes were competing for the substrate.

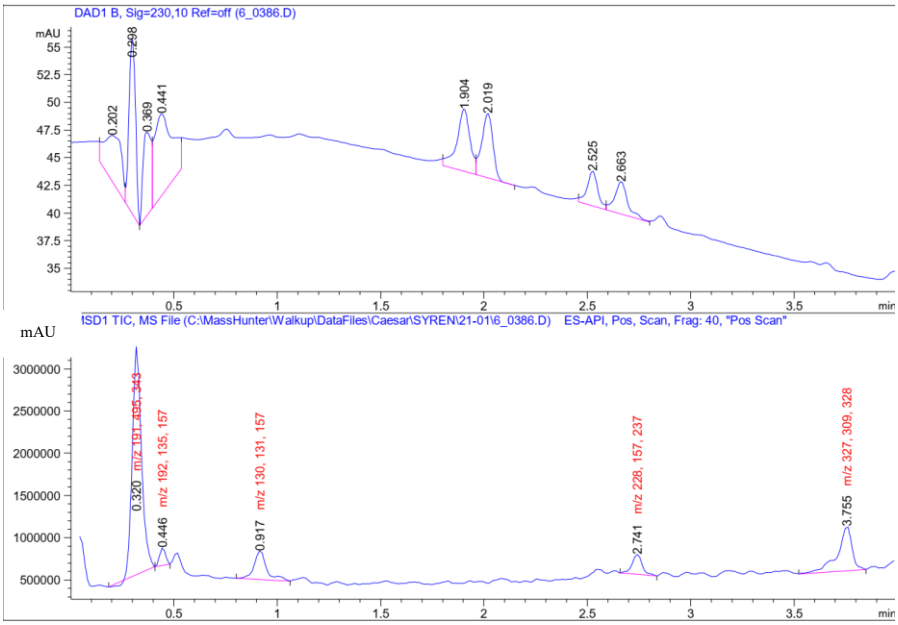


Figure 7. 46 HPLC chromatogram of P2 washed after 48 hours incubation with 15 µg LCC/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

P3 was degraded as well, even without the application of LCC, figure 7.47, in its dimers. Comparing the retention times and the molecular weight of these products with the results from P3 degradation using PETase, figure, those compounds were not equal. It appeared more probable that the PETase application results were the building dimers of P3 since their molecular weight was slightly different to the real one. In this case those were oligomers coming from hydrolysis and bond cleavage including crosslink and probably occurring in different points.

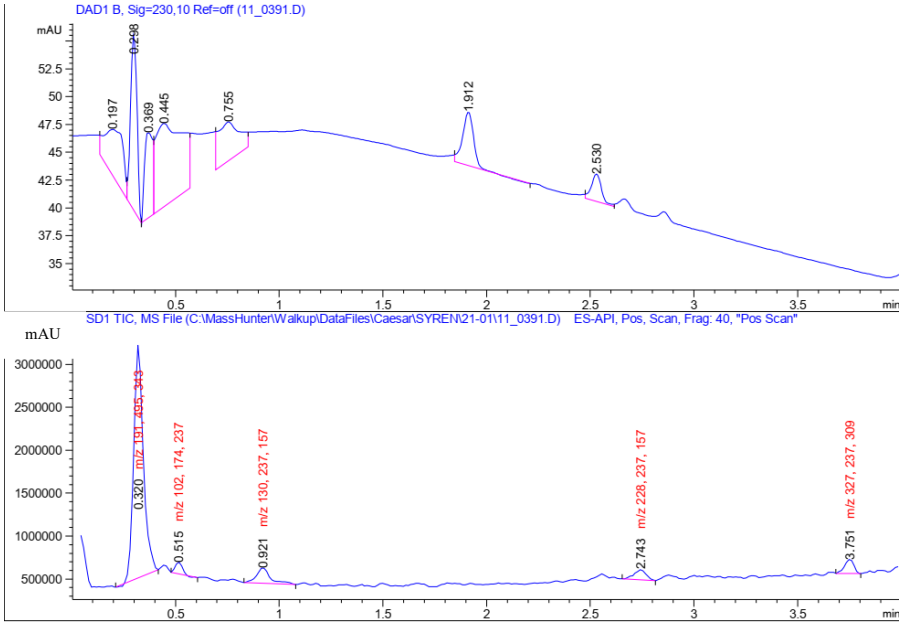


Figure 7. 47 HPLC chromatogram of P3 washed after 48 hours incubation with 0 µg LCC/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

$3.04*10^{-2}$ mg of monomer D were one of the results after the application of 1 µg LCC/5 mg polymer (retention time 0.445 min), figure 7.48, even though P3 was a copolymer, so it had a more complex structure. Despite that, also in this chromatogram it was not possible to detect any other monomers, including C, which HPLC result after the application of methanol, figure 7.49, did not show anything, apart from a peak at 1.900 min, in common with the control, indicating some dimers.

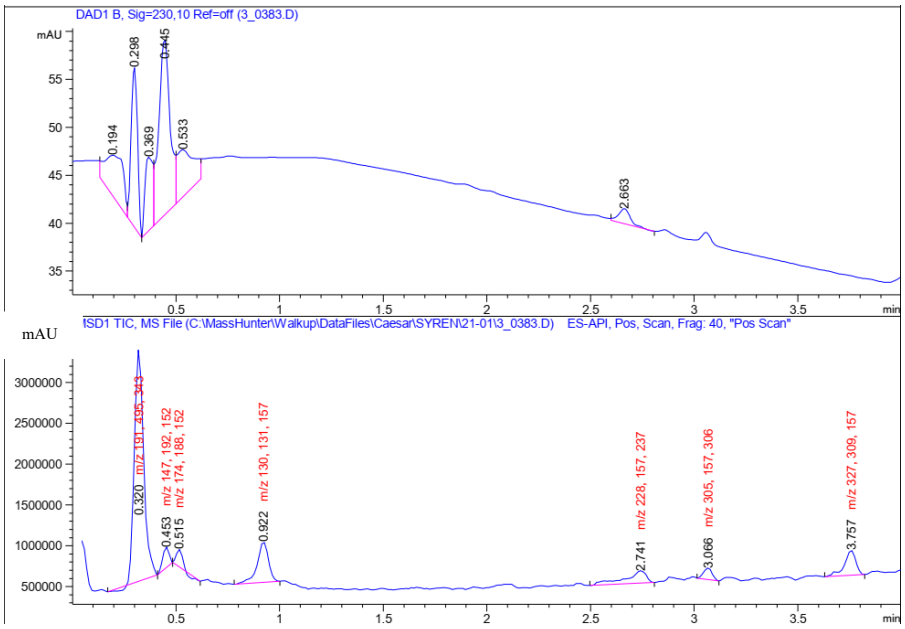


Figure 7. 48 HPLC chromatogram of P3 washed after 48 hours incubation with 1 µg LCC/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

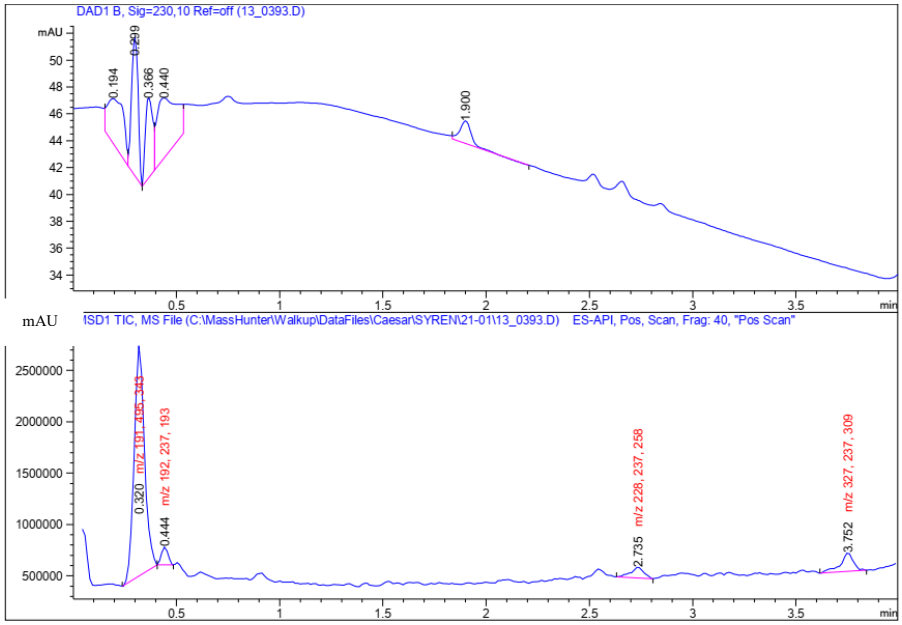


Figure 7. 49 HPLC chromatogram of P3 washed after 48 hours incubation with 1 µg LCC/5 mg polymer mixed with methanol, UV detector 230 nm (top) and MS detector (bottom)

Application of a higher concentration of LCC did not show any important differences with the control and, more important, confirmed that in this case the degradation stopped to the dimers and it was, hence, only due to the high temperature.

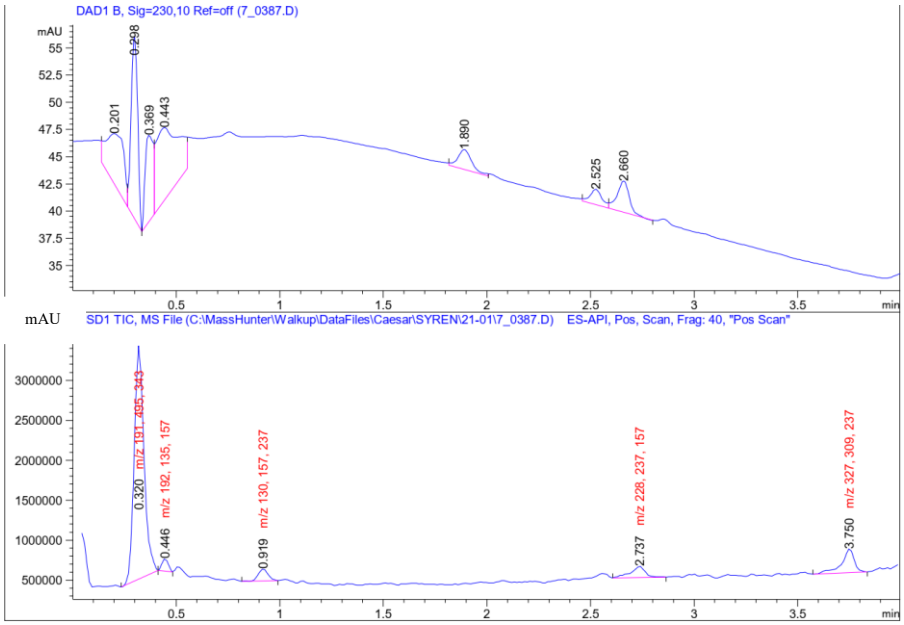


Figure 7. 50 HPLC chromatogram of P3 washed after 48 hours incubation with 15 µg LCC/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

HPLC chromatograms of P4 degradation using LCC are almost the same as for P3. The control, figure 7.51, showed the same dimers, apart from the last peak at 230 nm, 2.647 min.

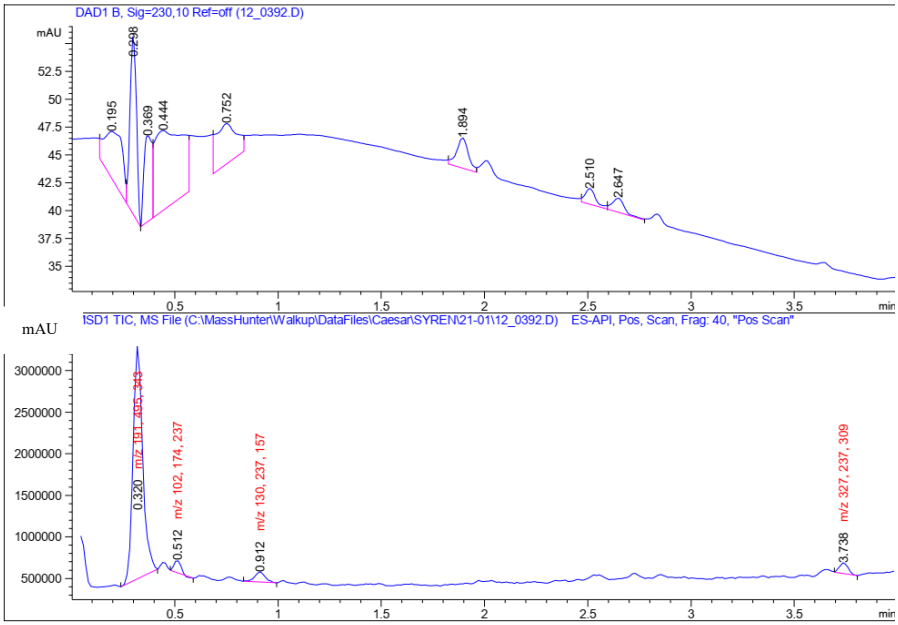


Figure 7. 51 HPLC chromatogram of P4 washed after 48 hours incubation with 0 µg LCC/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

Low concentration of LCC, chromatogram figure 7.52, hydrolysed P4 until monomer D emerged, but in low quantity, just $2.32*10^{-2}$ mg, confirming that monomer B was probably less attached to the enzyme active site: the branched double bond inhibited the catalytic reaction in some way.

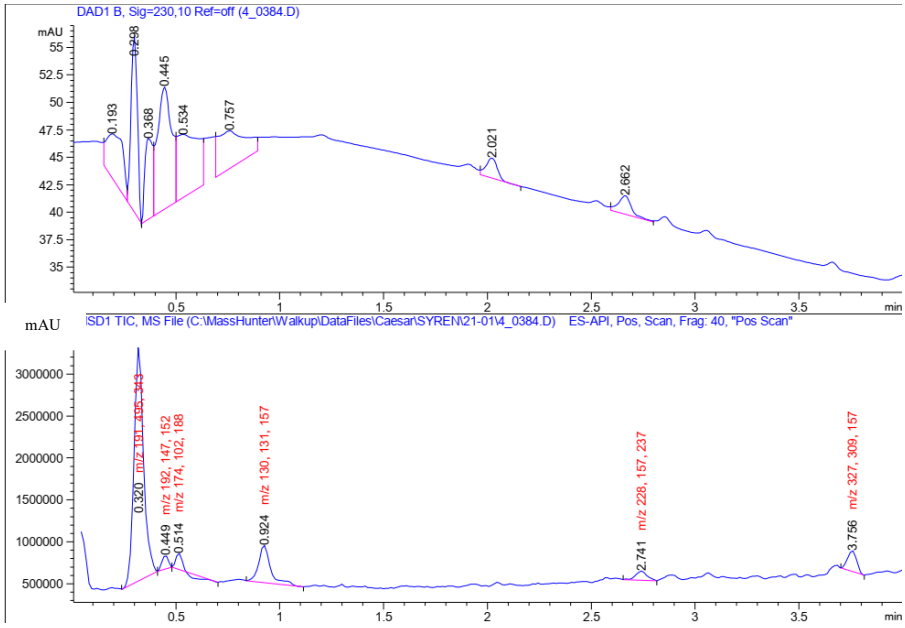


Figure 7. 52 HPLC chromatogram of P4 washed after 48 hours incubation with 1 µg LCC/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

Finally, high concentration of LCC, figure 7.53, continued the thermal degradation without starting a real catalytic reaction, as for all the other samples when this parameter was chosen.

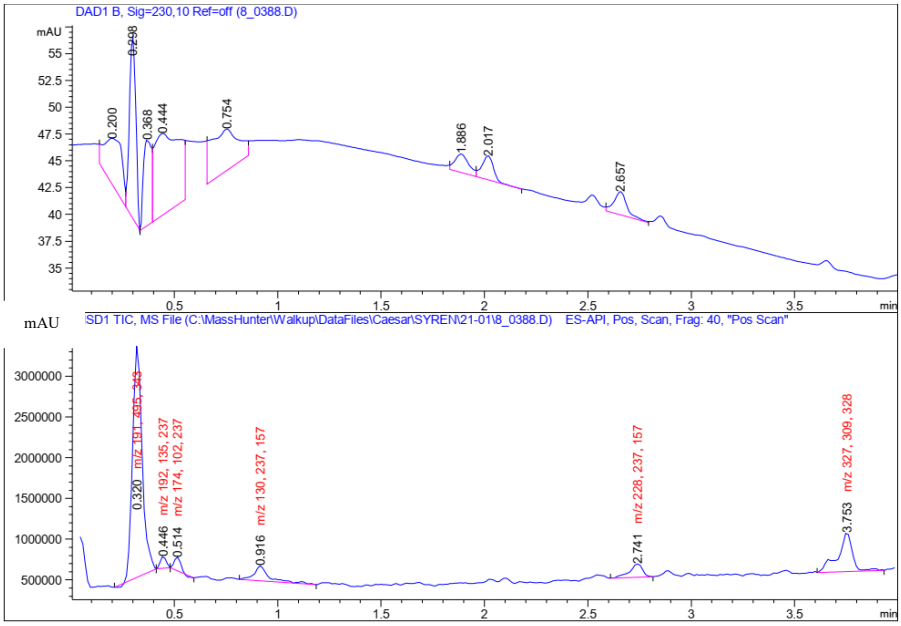


Figure 7. 53 HPLC chromatogram of P4 washed after 48 hours incubation with 15 µg LCC/5 mg polymer, UV detector 230 nm (top) and MS detector (bottom)

Considering the results, it seemed not useful to run a GC-MS measure, since the monomer retention time and molecular weight resembled the standard chromatogram and the dimers results were low and not comparable with the PETase ones.

To summarize the data of the LCC application after 24 and 48 hours a final chart is presented in figure 7.54 and 7.55. Only the results of low concentration enzyme and when an increase of the products occurred are shown. This choice was made to focus the attention on the most reliable results, the ones where the degradation was mainly due to the enzyme application and the hydrolysis continued in time.

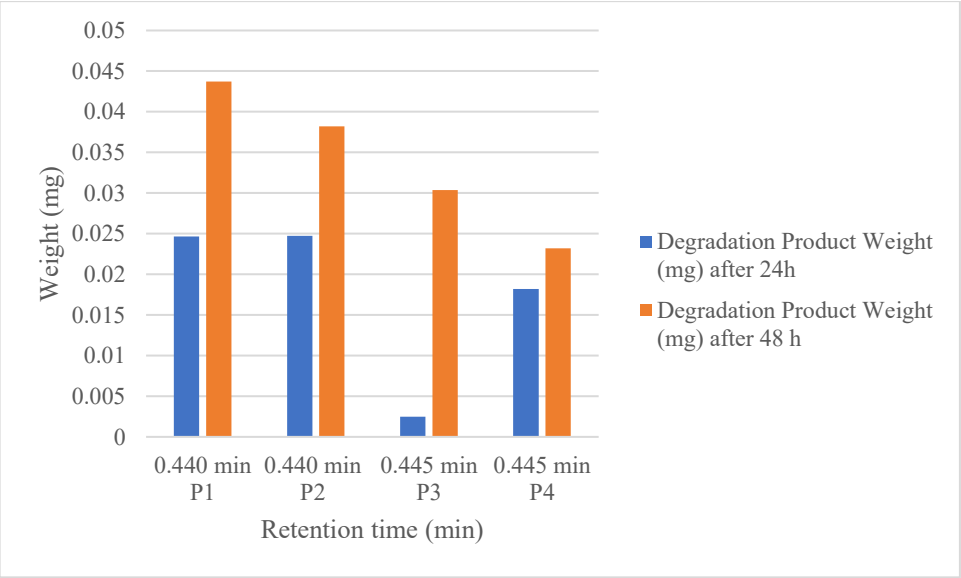


Figure 7. 54 Degradation Products, probably monomer D with retention time around 0.4 min, after 1 µg LCC/5 mg polymer application at different times

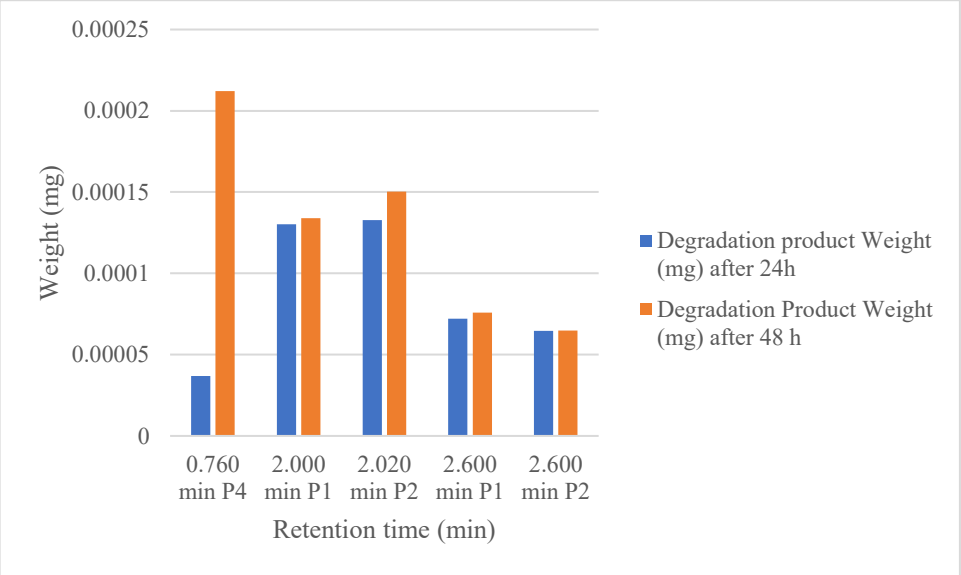


Figure 7. 55 Degradation Products, probably dimers, after 1 µg LCC/5 mg polymer application at different times

From the chart is clearly visible that P1 and P3 were more degraded compared to P2 and P4 respectively, probably because maleic acid was the most attached to the enzyme active site, compared to the itaconic acid. Moreover, the homopolymers P1 and P2 were more easily hydrolysed, probably for a structure less complex. It is clear, however, that LCC could hydrolase more compared to PETase, which only break the bonds of P3 and P4, semi aromatic compounds.

8. Conclusions

This study revealed that the possibility of using enzymes in degrading polyesters is an approach that should be taken into consideration. The theorical investigation suggested a possible a catalytic hydrolytic pathway for the considered molecules, comparing their characteristics to other bioplastics, especially PET (and its bio version, bio-PET), for the enzymatic degradation, and PLA, for the kinetic model and the affecting parameters. Focusing on PETase and LCC characteristics helped in understanding that PETase works better with aromatic substrates and has preferential final products, oligomers, while LCC is less selective towards specific polyesters.

Experimental results confirmed these assumptions, especially when it came to PETase, giving the highest degradation products for the semi-aromatic compounds and only small peaks for the aliphatic. Moreover, the compound showed high retention times, so they were probably dimers, a result that was confirmed also by their molecular weight through the GC MS chromatogram. Cutinase was, on the other hand, not only more effective, but it also gave more homogeneous results. The degradation occurred for all the samples and a monomer was produced in the same way for every one of them. The high quantity of products was probably enhanced by the higher temperature at which the incubation was run, but the type of molecules was definitively due to the enzyme activity, since in the control run only peaks at high retention times occurred.

Considering also that the studied molecules were in a crosslinked form and they contained sulfur bridges, not cleavable by the enzymes, this may have influenced the molecular weights, but also the activity itself in some cases. Despite that these characteristics made them amorphous, which may have helped the degradation considering the PLA hydrolysis model presented.

In both cases, anyway, a high concentration of the enzyme did not give appreciable results and the chromatograms followed the already started behaviour of the controls: no degradation products for PETase application and none of the monomer when LCC was used. The cause was a competitive action of the enzyme towards the substrate, in too low quantity to enter their active sites.

Moreover, it was discovered that the polymers should be pre-treated before the analysis, to wash all the charges and the protein on their surfaces: this facilitated the enzyme action. This came from the fact that non-washed samples when PETase was applied did not give any peaks.

Unfortunately, the SEM images for the polymers surface did not help the understanding of the process. The only conclusion that it can be said about that is that the hydrolysis occurred in some other zone of the polymer, but not on the surface.

The results showed that this was an important test to understand the value in using those enzymes, not only for the substrates they are known to degrade, but also in new applications. Once established, this technique can easily substitute all the chemical methods, since it is itself a type of solvolysis that does not involve any hard chemicals applications. Furthermore, especially for some methods like upcycling, the production of oligomers and dimers instead of monomers, as for PETase, is preferred to enhance the production of other materials based on those. However, some research must still be done, and some suggestion will be presented in the next chapter.

9. Future work

Future work regarding this subject will probably head towards a modification of the enzymes structure and on some other pre-treatment for the polymers, to make them more prone to the hydrolysis.

- The enzymes, especially PETase, must be more thermostable, to be incubated at higher temperature, as it was done with LCC, and exploit the thermal hydrolysis. Cui et al. [21] proposed a thermostable PETase, called Dura PETase, and it is only one of the many that are currently published or under investigation. PETase, when compared to cutinase, has a disulphide bridge more, which gives to it a higher thermostability, that, however, it is still not exploited at the best, but it can. Furthermore, to finish the degradation and produce the monomers, MHETase, the other enzyme responsible for PET hydrolysis, must be expressed as well and let it work in tandem with PETase.
- The crosslinks must be partly cleaved before the incubation with the enzyme. As it was described in chapter 4, this can be done using some chemicals: to be able to insert the all process in a circular economy vision, also this choice must be sustainable.

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11. Appendix A

11.1 Standards and polymers weight

11.1.1 Standards weight and buffer ratios

In the following table, 11.1, the contents of standards, buffer and methanol used to understand when to expect peaks and to draw the calibration curve are provided.

Table 11. 1 Standards weight and liquid ratios

Monomer	Weight (mg)	Buffer (ml)	Methanol (ml)	Ratio solid/liquid
Monomer D	**0**	**1**	**0**	**0**
Monomer D	1.32	0.264	0	0.005
Monomer D	7.25	0.75	0	0.010
Monomer D	1.54	0.103	0	0.015
Monomer D	2.16	0.108	0	0.020
Monomer D	3.70	0.148	0	0.025
Monomer A	0	1	0	0
Monomer A	5	1	0	0.005
Monomer A	10	1	0	0.010
Monomer A	15	1	0	0.015
Monomer A	20	1	0	0.020
Monomer A	25	1	0	0.025
Monomer B	0	1	0	0
Monomer B	5	1	0	0.005
Monomer B	10	1	0	0.010
Monomer B	15	1	0	0.015
Monomer B	20	1	0	0.020
Monomer B	25	1	0	0.025
Monomer C	0	1	0	0
Monomer C	7.5	0.5	1	0.005
Monomer C	10	0.5	1	0.007
Monomer C	22.5	0.5	1	0.015
Monomer C	30	0.5	1	0.020
Monomer C	37.5	0.5	1	0.025

11.1.2 Polymers and enzymes weight

In table 11.2 and 11.3 the weight of the used polymers, with the enzyme type and quantity can be found, for every type of treatment and concentration used.

Table 11. 2 PETase treatment polymers weight, enzyme, and buffer content (C1 means 1µgPETase/5 mg polymer, C2 means 15µgPETase/5 mg polymer, C0 means 0µgPETase/5 mg polymer)

Polymer	Weight (mg)	PETase content (µg)	Buffer volume (µl)
P1 C1 washed	4.35	0.87	998.99
P2 C1 washed	4.02	0.80	999.07
P3 C1 washed	3.31	0.66	999.23
P4 C1 washed	2.43	0.49	999.43
P1 C2 washed	3.94	11.82	986.26

P2 C2 washed	2.65	7.95	990.76
P3 C2 washed	5.08	15.24	982.79
P4 C2 washed	2.14	6.42	992.53
P1 C0 washed	4.28	0	1000
P2 C0 washed	6.06	0	1000
P3 C0 washed	4.23	0	1000
P4 C0 washed	4.02	0	1000
P1 C1 non washed	5.49	1.10	993.62
P2 C1 non washed	2.17	0.43	997.48
P3 C1 non washed	5.05	1.01	994.13
P4 C1 non washed	2.06	0.41	997.60
P1 C2 non washed	3.99	11.97	995.36
P2 C2 non washed	1.22	3.66	998.58
P3 C2 non washed	1.86	5.58	997.84
P4 C2 non washed	2.11	6.33	997.55
P1 C0 non washed	3.24	0	1000
P2 C0 non washed	3.26	0	1000
P3 C0 non washed	1.94	0	1000
P4 C0 non washed	1.91	0	1000

Table 11. 3 LCC treatment polymers weight, enzyme, and buffer content

Polymer	Weight (mg)	LCC content (µg)	Buffer volume (µl)
P1 C1 washed	9.95	1.99	997.51
P2 C1 washed	7.31	1.46	998.17
P3 C1 washed	1.24	0.25	999.69
P4 C1 washed	2.99	0.60	999.25
P1 C2 washed	13.36	40.08	949.90
P2 C2 washed	7.12	21.36	973.30
P3 C2 washed	1.82	5.46	993.18
P4 C2 washed	2.78	8.34	989.58
P1 C0 washed	8.50	0	1000
P2 C0 washed	5.77	0	1000
P3 C0 washed	1.03	0	1000
P4 C0 washed	2.08	0	1000

11.2 Degradation products

In the following tables the results after the application of both enzymes, in terms of area (and weights, using equation 7.1) and retention times.

Table 11.4 shows the results of PETase application after 48 hours, tables 11.5 and 11.6 the ones of LCC use after 48 hours and their relative 24 hours (the empty rows mean that there was no correspondence). All the analysed samples have been washed before.

Table 11. 4 Resulting areas, weights and retention times of peaks after PETase 48h incubation analysed through HPLC

48 h incubation samples	Retention time (min)	Area (mAU*s)	Weight (mg)
P1 C2	2.336	12.48722	8.35E-05
P1 C2	2.989	5.45732	3.65E-05
P2 control	2.783	12.01387	8.04E-05
P2 C1	2.344	13.39309	8.96E-05
P2 C1	2.789	7.74707	5.18E-05
P2 C2	2.346	8.0749	5.4E-05
P3 C1	2.344	36.67151	0.000245
P3 C1	2.997	17.82213	0.000119
P3 C1	3.308	5.90693	3.95E-05
P4 C1	2.338	24.03816	0.000161
P4 C1	2.990	11.60324	7.76E-05

Table 11. 5 Resulting areas, weights and retention times of peaks after LCC 48h incubation analysed through HPLC

48 h incubation samples	Retention Time (min)	Area (mAU*s)	Weight (mg)
P1 control	0.437	43.40283	0.018962
P1 control	1.897	11.06481	7.4E-05
P1 control	2.014	20.62397	0.000138
P1 control	2.655	12.08373	8.08E-05
P1 C1	0.08	9.09247	6.08E-05
P1 C1	0.441	100.0558	0.043713
P1 C1	0.528	35.95146	0.00024
P1 C1	0.948	32.13897	0.000215
P1 C1	2.011	20.03491	0.000134
P1 C1	2.659	11.3438	7.59E-05
P1 C1	3.053	5.28308	3.53E-05
P1 C2	0.443	66.32198	0.028975
P1 C2	0.752	32.10001	0.000215
P1 C2	1.893	35.6824	0.000239
P1 C2	2.018	50.16518	0.000336
P1 C2	2.521	15.61877	0.000104
P1 C2	2.659	19.11324	0.000128
P2 control	0.443	41.1241	0.017967
P2 control	0.755	5.10398	3.41E-05
P2 control	1.909	62.22483	0.000416
P2 control	2.026	17.73197	0.000119
P2 control	2.532	26.33477	0.000176
P2 control	2.665	10.35173	6.92E-05
P2 C1	0.444	87.44279	0.038203
P2 C1	0.533	26.47402	0.000177
P2 C1	2.023	22.46418	0.00015
P2 C1	2.663	9.69539	6.48E-05

P2 C1	3.058	6.16271	4.12E-05
P2 C2	0.441	44.2604	0.019337
P2 C2	1.904	24.94812	0.000167
P2 C2	2.019	21.76341	0.000146
P2 C2	2.525	11.31217	7.57E-05
P2 C2	2.663	12.74442	8.52E-05
P3 control	0.445	66.12247	0.028888
P3 control	0.755	25.03671	0.000167
P3 control	1.912	17.48945	0.000117
P3 control	2.53	8.30912	5.56E-05
P3 C1	0.445	69.49765	0.030363
P3 C1	0.533	27.08897	0.000181
P3 C1	2.663	6.12796	4.1E-05
P3 C2	0.443	49.24802	0.021516
P3 C2	1.89	8.32676	5.57E-05
P3 C2	2.525	5.70265	3.81E-05
P3 C2	2.66	11.74113	7.85E-05
P4 control	0.444	65.60763	0.028663
P4 control	0.752	25.52051	0.000171
P4 control	1.894	10.78702	7.22E-05
P4 control	2.51	5.03998	3.37E-05
P4 control	2.647	5.03271	3.37E-05
P4 C1	0.445	53.14442	0.023218
P4 C1	0.534	40.67184	0.000272
P4 C1	0.757	31.70977	0.000212
P4 C1	2.021	6.81176	4.56E-05
P4 C1	2.662	7.25833	4.85E-05
P4 C2	0.444	63.46294	0.027726
P4 C2	0.754	36.69733	0.000245
P4 C2	1.886	8.05924	5.39E-05
P4 C2	2.017	8.44601	5.65E-05
P4 C2	2.657	9.52311	6.37E-05

Table 11. 6 Resulting areas, weights and retention times of peaks after LCC 24h incubation analysed through HPLC

24 hours samples	Time	Area	Weight
P1 control	0.439	43.40672	0.018964
P1 control			0
P1 control	2.031	18.43282	0.000123
P1 control	2.675	8.4137	5.63E-05
P1 C1			0
P1 C1	0.443	56.4039	0.024642
P1 C1			0
P1 C1			0
P1 C1	2.043	19.46165	0.00013
P1 C1	2.684	10.76998	7.2E-05
P1 C1	2.869	8.00979	5.36E-05
P1 C2	0.439	42.59296	0.018608
P1 C2			0
P1 C2			0
P1 C2	2.032	19.34862	0.000129
P1 C2			0
P1 C2	2.672	9.03559	6.04E-05

P2 control	0.443	44.59423	0.019483
P2 control			0
P2 control			0
P2 control	2.035	15.89799	0.000106
P2 control			0
P2 control	2.671	6.97065	4.66E-05
P2 C1	0.446	56.59304	0.024725
P2 C1			0
P2 C1	2.045	19.83611	0.000133
P2 C1	2.681	9.64938	6.45E-05
P2 C1	2.868	6.55364	4.38E-05
P2 C2	0.445	50.46446	0.022047
P2 C2			0
P2 C2			0
P2 C2			0
P2 C2	2.672	9.92495	6.64E-05
P3 control	0.446	42.58826	0.018606
P3 control			0
P3 control			0
P3 control			0
P3 C1	0.445	5.71187	0.002495
P3 C1			0
P3 C1			0
P3 C2	0.445	50.46446	0.022047
P3 C2			0
P3 C2			0
P3 C2	2.672	9.92495	6.64E-05
P4 control	0.445	44.15516	0.019291
P4 control			0
P4 control			0
P4 control			0
P4 control			0
P4 C1	0.445	41.62497	0.018186
P4 C1			0
P4 C1	0.76	5.4991	3.68E-05
P4 C1	2.043	6.48544	4.34E-05
P4 C1	2.682	5.92356	3.96E-05
P4 C2	0.444	49.00078	0.021408
P4 C2			0
P4 C2			0
P4 C2	2.026	6.89069	4.61E-05
P4 C2	2.67	7.07331	4.73E-05

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