BIOREFINERY FOR BIOPOLYMERS: NEW TOOLS FOR BIOMATERIALS PRODUCTION, DEGRADATION AND SUSTAINABLE FUNCTIONALIZATION

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Riassunto

I polimeri sono tra le prime sostanze con cui l'uomo è venuto in contatto fin dai primordi della sua storia. La lunghezza delle catene, la tipologia, il numero di monomeri e la loro disposizione determinano le caratteristiche di questi composti, che rappresentano una classe di materiali ubiquitari nella vita quotidiana. Le plastiche, un tipo particolare di polimeri, derivano in larga parte, ancora oggi, dalla chimica del petrolio. La necessità di salvaguardare l'ecosistema, unita alle problematiche economico-politiche legate ai combustibili fossili ha fortemente spinto la ricerca verso la messa a punto di processi innovativi ed ecocompatibili per prodotti e materiali sostenibili.
I biopolimeri, definiti come polimeri prodotti a partire da biomasse rinnovabili e/o da organismi viventi, rappresentano una concreta alternativa ecologica a quelli derivanti da fonti fossili. Tali biopolimeri oltre ad essere intrinsecamente rinnovabili spesso presentano caratteristiche di biodegradabilità e biocompatibilità. Il limite alla diffusione di tali bioplastiche, però, risiede nel fatto che le conoscenze attuali non sono tali da rendere la loro produzione economicamente competitiva.
Argomento di questo lavoro di dottorato è stata **la messa a punto di nuovi sistemi e tecnologie sostenibili per la sintesi, la funzionalizzazione e la degradazione di biopolimeri.** Particolare attenzione è stata rivolta allo sfruttamento di reflui di processi industriali come substrati per la loro produzione al fine di inquadrare l'intero processo in un più ampio scenario di bioraffineria ed economia circolare.

Tra i vari biopolimeri, una classe che ha iniziato ad affacciarsi sul mondo del mercato è quella dei Polidrossialcanoati (PHA). I PHA sono poliesteri microbici derivanti dalla condensazione di unità idrossiaciliche. Questi sono classificati in *short chain legth* (scl) e *medium chain length* (mcl) in funzione del numero di atomi di carbonio dell'unità monomerica. Le proprietà fisiche e meccaniche di tali polimeri sono strettamente correlate alla loro composizione. Tale peculiarità rende questa classe di biopolimeri, un valido candidato per affiancare e/o sostituire le plastiche derivanti da combustibili fossili. I PHA, che in natura hanno la funzione di riserva energetica, sono accumulati come risposta a condizioni di stress quali la mancanza di particolari nutrienti ed ossigeno.
Nel percorso di dottorato sono state investigate le performances produttive di sistemi ricombinanti di *Escherichia coli* basati sull'operone biosintetico di PHA da *Bacillus cereus* 6E/2. L'interesse verso questi sistemi nasce dalla scarsa letteratura riguardante la produzione ricombinante di PHA catalizzata da proteine appartenenti a specie di *Bacillus* (classe IV), e dalle potenzialità che possono celarsi dietro enzimi che hanno mostrato, nei sistemi nativi, un'ampia specificità di substrato. Sono riportati, infatti, esempi di *Bacilli* in grado di produrre non sono omopolimeri di PHB ma anche di incorporare monomeri a catena più lunga quali il 3-idrossiesanoato (3HHx). La prima parte del lavoro ha riguardo la costruzione del sistema esprimente i tre geni (*phaR*, *phaB*, *phaC*) codificante per le 3 proteine necessarie e sufficienti per la produzione di biopolimeri a partire da fonti di carbonio lipidiche. Una volta messo a punto il protocollo di estrazione di DNA genomico dal ceppo di *B. cereus* selezionato ed amplificata la zona di interesse, dall'analisi di sequenziamento è stata rivelata un'ambiguità in merito l'inizio della regione codificante la prima proteina dell'operone: PhaR. Tale incertezza ha portato alla definizione di due possibili varianti: sPhaR (*short*), LPhaR (*Long*). Sono stati costruiti sistemi esprimenti entrambe le versioni della prima proteina, complementati con le restanti proteine necessarie alla produzione di PHA. Il sistema esprimente la versione *short* della proteina si è rivelato come il più performante ed è stato caratterizzato crescendolo in presenza di diverse

fonti di carbonio lipidiche (sali di acidi grassi di diversa lunghezza, oli vegetali, oli vegetali esausti). Lo studio della composizione dei polimeri prodotti ha portato alla luce una particolare tendenza del ceppo costruito (*sPha* definito poi *LipoA*) nell'incorporare monomeri a catena lunga ottenendo copolimeri PHB-co-PHHx con un elevato contenuto di 3HHx (**Capitolo 2.1**).
Nonostante le interessanti prospettive derivanti da tale peculiarità, le rese di produzione del sistema *LipoA* non erano soddisfacenti nelle condizioni di crescita/produzione analizzate. Al fine di ottenere un maggior quantitativo di biopolimero ed approcciarne la funzionalizzazione sono stati testati diversi terreni di crescita. Una volta selezionate le condizioni che hanno mostrato il migliore bilancio tra crescita microbica e produzione di biopolimero, il sistema *LipoA* è stato sottoposto ad un processo di ingegnerizzazione che ha previsto la sostituzione del sistema atto alla regolazione della trascrizione dell'operone (*LipoB*) e la sostituzione del background metabolico del ceppo ospite (*LipoC*). Entrambe le variabili si sono mostrate determinanti nelle rese di PHA prodotto evidenziando come la combinazione promotore *tac* e ceppo BL21(DE3) (*LipoB*) sia la più efficace nelle condizioni analizzate. L'analisi GPC dei polimeri prodotti dal sistema *LipoB* ha mostrato come le diverse condizioni di crescita possano influenzare fortemente il peso molecolare dei biopolimeri accumulati. Selezionata una condizione, la produzione è stata verificata anche in fermentatori mostrando un incremento delle rese di produzione rispetto alle analoghe crescite condotte in beuta. Il polimero prodotto è stato caratterizzato ed ha mostrato un basso grado di cristallinità ed un notevole carattere idrofobico. Al fine di aumentare il range di applicabilità del biopolimero prodotto ne è stata approcciata la funzionalizzazione enzimatica. La preparazione commerciale di lipasi B da *Candida antartica* immobilizzata (CaLB) è stata selezionata per catalizzare il coupling delle estremità carbossiliche ed ossidriliche del PHA prodotto rispettivamente con polietilenglicole (PEG) e dimetilitaconato (DMI). La funzionalizzazione con il polimero biocompatibile ha modificato l'idrofilicità del PHA ampliando la gamma dei possibili settori di applicazione, mentre il legame estereo con acido itaconico ha conferito al biopolimero un doppio legame terminale facilmente funzionalizzabile con reazioni di "*click chemistry*". La reazione con il derivato dell'acido insaturo ha portato alla formazione di specie di PHA ad alto peso molecolare frutto della condensazione di unità a più basso peso molecolare unite da una molecola di DMI. Al fine di ottenere un PHA con le caratteristiche attribuite sia da PEG che DMI sono state condotte anche reazioni contemporanee e sequenziali tra i tre substrati. In questo caso, per tutte le specie PHA-DMI-PEG non è stata osservata la formazione di PHA ad alto peso molecolare, probabilmente a causa di un attacco nucleofilo portato dal PEG al legame formatosi in una prima fase tra PHA e DMI. In tutti i derivati ottenuti non è stata riscontrata una diminuzione del peso molecolare del polimero di partenza espressione dalla validità dell'approccio enzimatico per la funzionalizzazione soft di biopolimeri. Le condizioni di reazione, infatti, assicurano l'integrità del biopolimero di partenza evitando degradazione (**Capitolo 2.2**).

Un analogo approccio enzimatico è stato applicato anche per la sintesi di oligoesteri. In questo caso è stata selezionata la cutinasi 1 da *Thermobifida cellulosilytica* (Thc_Cut1). L'enzima, prodotto per via ricombinante in *E. coli* con un opportuno *tag* di istidine, è stato immobilizzato su tre diversi supporti con polarità diversa forniti dall'azienda svedese EnginZyme**:** Opal, Coral e Amber. Il legame tra l'enzima ed il supporto è stato assicurato con un innovativo metodo di immobilizzazione non

tossico, basato sull'interazione tra His-tag e ioni di Fe (III) che fungono da chelante. Le tre preparazioni così ottenute di enzima immobilizzato sono state caratterizzate in reazioni di sintesi in assenza di solvente con diversi coppie di substrati. Nello specifico sono stati analizzati dioli da 4 a 8 atomi di carbonio in combinazione con diesteri metilici di acidi bicarbossilici da 4 a 8 atomi di carbonio. Contrariamente a quanto mostrato dalla lipasi maggiormente studiata per questo tipo di applicazioni sintetiche (CaLB), Thc_Cut1 ha mostrato una preferenza per la combinazione diolo-estere C4-C6 per tutti i supporti analizzati. Nel caso dell'enzima immobilizzato sul supporto Amber è stata osservata una resa di conversione del monomero fino al 78% e produzione di oligomeri butiladipato con peso molecolare Mw di 878. È stata valutata, inoltre, la possibilità di riciclare le preparazioni enzimatiche. I dati ottenuti per cicli di 24 h di reazione ed allontanamento del prodotto ottenuto senza l'utilizzo di solventi, hanno mostrato una ritenzione di attività superiore al 90%. Così come osservato per CalB l'utilizzo di solventi organici causa una marcata diminuzione dell'attività dell'enzima. I risultati ottenuti rappresentano una valida alternativa enzimatica per la sintesi di oligoesteri funzionali per la produzione di nanoparticelle ed per la sintesi di pre-polimeri pronti alla reticolazione *in situ* per applicazioni di coating (**Capitolo 2.3**).

Thc_Cut1 è stato studiato non solo per reazioni di sintesi, ma anche per la reazione inversa di idrolisi. Sono state analizzate le potenzialità dell'enzima espresso nell'ospite *Pichia pastoris* in forma *wild type* ed in due forme mutanti. Le due varianti sono state disegnate al fine di eliminare i siti di glicosilazione. Non sono state osservate particolari differenze nella produzione in lievito tra le tre proteine. Gli enzimi purificati sono stati caratterizzati per la degradazione di poliesteri aromatici (PET) ed alifatici (PBS, PHBV). L'analisi HPLC dei prodotti di idrolisi del PET ha rivelato un'efficacia simile tra le tre varianti proteiche. Per la caratterizzazione delle performance idrolitiche contro substrati alifatici è stata selezionata la variante non glicosilata che ha mostrato le rese di purificazione più elevate: Thc_Cut1_koST. Le prime analisi sono state condotte sui polimeri in polvere mostrando, per entrambi gli enzimi, una maggiore efficacia nei confronti del PBS rispetto il copolimero di PHBV. In particolare, la versione mutata ha portato ad una concentrazione dei prodotti di rilascio quasi due volte maggiori rispetto a quanto ottenuto usando l'enzima *wild type*. La stessa differenza è stata osservata anche per le idrolisi condotte sui polimeri in forma di film sottili. In questo caso la diminuzione di peso dei campioni trattati con i due enzimi è sensibilmente diversa: dopo 96 h di reazione è del 41% per l'enzima *w.t.* mentre 93% per il mutante. L'analisi SEM dei campioni ha mostrato un netto cambiamento morfologico delle superfici dei film trattati con entrambi gli enzimi (**Capitolo 2.4**).

Un'altra parte importante di questo lavoro ha riguardo lo sviluppo di nuovi sistemi per la produzione di PHA a partire da reflui industriali con particolare attenzione alla filiera produttiva del biodiesel (**Sezione 3**). Tale approccio ha permesso di inquadrare a pieno la produzione di biopolimeri in un'ottica di bioraffineria ed economia circolare. Esperimenti di produzione di PHA sono stati condotti su: i) oli vegetali esausti (oli di frittura, WFO) in quanto potenziali substrati per la produzione di biodiesel e ii) glicerolo quale prodotto di scarto della reazione di transesterificazione alla base della produzione del biocarburante.
Gli oli vegetali esausti rappresentano un rifiuto complesso ed eterogeneo. Gli oli esausti attualmente raccolti derivano principalmente da aziende del settore della

ristorazione e solo in minima parte da produzioni casalinghe. Circa il 90% degli oli raccolti in Europa è destinato alla produzione di biodiesel. Il crescente interesse verso la produzione di questo biocarburante ha portato alla nascita di un vero e proprio mercato per quello che prima era un rifiuto destinato allo smaltimento. Il prezzo degli oli esausti è funzione di diversi fattori ed il contenuto di acidi grassi liberi (FFA) è il principale parametro che influenza il valore sul mercato di un olio esausto. Maggiore è il contenuto di acidi grassi in un olio, minore sarà il suo valore. Tale proporzionalità inversa è riconducibile alle difficoltà legate alla conversione in biodiesel di oli con un elevato contenuto di acidi grassi. Per oli con un contenuto di acidi grassi superiore al 5 %, infatti, è richiesto un pretrattamento con acido per ottenere rese di conversione in biodiesel soddisfacenti. Lo step di pretrattamento è economicamente vantaggioso fino a concentrazioni di acidi grassi prossime al 10%. Superata tale soglia, l'olio esausto perde ogni valore e deve essere smaltito.
Un Bioprocesso basato sull'utilizzo di tali oli come substrato per una crescita microbica, ne riduce il contenuto in acidi grassi e consente la produzione di biomateriali ad elevato valore aggiunto rappresenta una via per valorizzare un refluo altrimenti destinato alla discarica.
I primi esperimenti sono stati condotti con oli derivanti da produzioni casalinghe o forniti da piccole aziende del settore agroalimentare. Due diversi sistemi ricombinanti (*LipoA* e *A-sPha* in seguito *OmniA*) sono stati testati per la conversione degli acidi grassi liberi dei reflui in bioplastiche. I sistemi di *E. coli* selezionati avevano diverse potenzialità biosintetiche: in un caso è possibile la conversione in biopolimero (mcl-PHAs) solo di fonti di carbonio lipidiche, nell'altro è possibile trasformare qualsiasi tipo di fonte di carbonio in scl-mcl-PHA. La produzione di PHA è stata osservata solo a seguito di uno step di estrazione acquosa dei reflui selezionati. Nonostante le basse rese in termini di biopolimeri prodotti, i sistemi hanno mostrato interessanti potenzialità in termini di riduzione del contenuto di acidi grassi liberi degli oli esausti (**Capitolo 3.2**).

I dati ottenuti hanno spinto all'ingegnerizzazione del sistema al fine di ottimizzare le rese in bolipomero. Tale processo ha previsto lo studio delle performance di: i) nuovi terreni in grado di ovviare all'alta variabilità degli oli esausti e supportare una maggiore produzione di PHA, ii) un sistema risultato essere più adatto alla produzione ricombinante di PHA (*LipoB*). Terminata la fermentazione microbica (72 h) gli oli sono stati recuperati e dopo la caratterizzazione in termini di contenuto di acidi grassi liberi sono stati sottoposti alla reazione di transesterificazione per la produzione di biodiesel. I dati ottenuti hanno portato alla *proof of concept* per il bioprocesso proposto che, riducendo il contenuto di acidi grassi liberi, ha reso gli oli recuperati al termine della fermentazione direttamente convertibili in biodiesel. Ancora una volta, però, i sistemi ricombinanti selezionati non si sono rivelati efficaci nel produrre soddisfacenti quantità di biopolimero.
La fase successiva ha previsto lo studio delle potenzialità di sistemi nativi per il bioprocesso proposto. Il principale limite all'applicabilità di microorganismi in questo contesto è riconducibile alla presenza di lipasi extracellulari che possono aumentare il contenuto di FFA a seguito dell'idrolisi di trigliceridi dell'olio. Lo studio dei ceppi microbici caratterizzati per la produzione di PHA ha portato alla selezione del ceppo di *Pseudomonas resinovorans* NRL B-2649. In tale ceppo, infatti, è riportata la presenza di un unico gene codificante per una lipasi di secrezione. Inoltre era già stato riportato in letteratura lo sviluppo di un mutante privo di tale attività idrolitica a seguito del knock-out di tale gene. Una volta ottenuti i due ceppi di *P. resinovorans*,

le loro performance in termini di produzione di PHA e riduzione degli acidi grassi liberi sono state analizzati su terreno contenente oli vegetali esausti con un contenuto di FFA superiore al 10%. I due ceppi si sono dimostrati pressoché equivalenti dopo 72 h di crescita arrivando a produrre PHA fino ad 1.3 g/L (circa 10 volte rispetto quella osservata in *E. coli*) e restituendo oli direttamente convertibili in biodiesel. Al fine di aumentare la produttività di biopolimero è stata aggiunta una fonte di carbonio in modo da indurre lo squilibrio di nutrienti nel terreno, utile all'attivazione del meccanismo di produzione dei PHA. Il glicerolo è stato selezionato come substrato a basso costo in quanto sottoprodotto del processo di produzione del biodiesel. L'aggiunta della fonte di carbonio extra si è dimostrata efficace a concentrazioni pari 0.2% con un aumento del 15% della resa in PHA. Non è stato osservato un ulteriore aumento della produzione di biopolimero per una concentrazione più elevata di glicerolo (0.8%).
I dati registrati al termine delle 72 h di fermentazione mostravano per entrambi i ceppi un contenuto di acidi grassi ancora considerevole (4-5%). Con l'intento di massimizzare la riduzione degli FFA un secondo step di fermentazione è stato implementato utilizzando gli oli recuperati dopo il primo step di 72 h per un totale di 144 h complessive. In questo caso le differenze tra i due ceppi sono state considerevoli. Nel caso del ceppo mutato l'olio ha mostrato una concentrazione finale di acidi grassi sempre inferiore al 1% e rese di transesterificazione superiore al 80%. Le stesse rese di conversione in biodiesel sono state ottenute anche con una concentrazione di catalizzatore dimezzata rispetto quella utilizzata per gli esperimenti precedenti, ottenendo un netto guadagno sia economico che ambientale. Nel caso del ceppo *wild type*, al contrario, la concentrazione di acidi grassi nell'olio dopo il secondo step di fermentazione è risultata molto variabile. In alcuni casi è stata registrata una concentrazione di FFA addirittura maggiore rispetto quella dell'olio non fermentato (15% Vs 10%). Tale andamento è stato ricondotto all'attivazione della secrezione della lipasi che non avviene durante le prime 72 h di fermentazione a causa degli acidi grassi liberi ancora presenti nel mezzo in discrete quantità.
Il diverso comportamento dei due sistemi è stato osservato anche in termini di produzione di PHA: il ceppo *wt* ha mostrato una produzione di PHA fino a 4 volte maggiore del ceppo mutante.
I due sistemi hanno mostrato caratteristiche peculiari che li rendono alternativamente utilizzabili nel bioprocesso disegnato per l'ottenimento dei 2 bioprodotti di interesse (oli convertibili in biodiesel e biopolimeri) a seconda del target desiderato (**Capitolo 3.3**).

Ulteriore argomento di studio è stata la costruzione di nuovi sistemi ricombinanti, *OmniB* ed *OmniC* analoghi ai sistemi *Lipo* presentati nel **Capitolo 2.2**, in grado di convertire una più ampia gamma di substrati in PHA e non più solo lipidi. Lo sviluppo di questi nuovi ceppi permette non solo di espandere la varietà di polimeri prodotti ma anche di reintrodurre il glicerolo grezzo sottoprodotto della produzione di biodiesel in un processo produttivo. I sistemi esprimenti gli stessi enzimi biosintetici sono stati analizzati su due diversi terreni, capaci di indurre la produzione di polimeri diversi. L'influenza del sistema di regolazione dell'espressione dei geni ricombinanti e del background metabolico sulla produzione di biopolimeri è stata analizzata in maniera analoga a quanto fatto per i sistemi *Lipo*.
Così come osservato per i sistemi in grado di produrre PHA da fonti lipidiche (**Capitolo 2.2**), anche in questo caso le due variabili analizzate hanno sensibili effetti sulla produttività di PHA. Entrambi i sistemi in BL21(DE3) (*OmniA*, *OmniB*) non

hanno mostrato produzione di PHA quando cresciuti senza il supporto di fonti di carbonio lipidiche. La β-chetotiolasi eterologa (PhaA), essenziale per la produzione di monomeri a partire da Acetil-CoA, è risultata in grado di espletare la propria funzione solo nel ceppo di *E. coli* LS5218 (*OmniC*). Per verificare l'effetto dell'enzima ricombinante al netto del contributo apportato dalla riportata attività β-chetotiolasica endogena del ceppo LS5218, i dati ottenuti con il sistema *OmniC* sono stati confrontati con quelli del sistema *LipoC*. I valori di produttività doppi in OmniC rispetto a *LipoC* dimostrano l'efficacia della PhaA eterologa.
In tutte le condizioni analizzate non è stata osservata una produzione soddisfacente di biopolimero. Per aumentare le rese in biopolimero è stato seguito un approccio di ingegneria metabolica identificando NADPH come un possibile target. Tale molecola, infatti, è un cofattore di uno degli enzimi biosintetici essenziali per la produzione di PHA. Al fine di massimizzare la concentrazione di NADPH disponibile, le crescite sono state condotte anche in presenza di glutammato (substrato in grado di aumentare la disponibilità di NADPH).
Per *OmniC*, combinazione promotore *tac* in LS5218, è stato osservato l'effetto positivo del glutammato arrivando ad un incremento della produzione di PHA fino al 50%. La criticità relativa alla disponibilità del NADPH è stata affrontata non solo aumentando le concentrazioni disponibili, ma anche andando ad agire sull'affinità di legame dell'enzima per il proprio cofattore. Lo studio di un'altra classe di reduttasi (flavoreduttasi), ed il confronto di sequenza e strutture 3D di β-chetoreduttasi ha portato al riconoscimento dei residui critici per l'interazione con NADPH. È stato quindi possibile il design di due mutanti potenzialmente più adatti alla produzione ricombinante di PHA (**Capitolo 3.4**).

Summary

Biopolymers are attractive “green” alternatives to conventional petroleum-based plastics, however, their sustainable exploitation is hampered by the high production costs. In this PhD thesis, an *Escherichia coli* recombinant system (*LipoA*) was constructed to allow production of Polyhydroxyalkanoates (PHAs). The system was engineered with a newly isolated PHAs biosynthetic operon from *Bacillus cereus* 6E/2 and tested for PHAs production on different carbon sources. Results highlighted the *LipoA* peculiar specificity to drive the incorporation of 3-hydroxyhexanoate monomers (>40%), whatever was the supplied fatty acid. To increase polymer production, media optimization and system engineering were applied. In this frame, two new PHA producing systems were developed considering i) the expression levels of the recombinant PHA bio-synthetic proteins (*LipoB*) and ii) the "host metabolic background" (*LipoC*) yielding to a 6-fold increment of mcl-PHA yields. Polymers were characterized revealing a low grade of crystallinity and hydrophobic features. To enhance biopolymer properties and expand fields of applicability, PHAs were enzymatically functionalized. Commercial lipase B from *Candida antarctica* (CaLB) was able to catalyse coupling of PHA with dimethyl itaconate (DMI) as well as with polyethylene glycol (PEG). The obtained functional hydrophilic biopolymers open new perspectives for application of PHAs in the biomedical field thanks to the possibility of easy coupling of bioactive compounds on the lateral C=C of DMI and to the enhanced hydrophilicity conferred by the PEG moieties. The enzymatic strategy was also applied for sustainable synthesis of oligoesters. Catalytic potential of immobilized *Thermobifida cellulosilytica* cutinase 1 (Thc_Cut1) was investigated. Three different carriers, linked to the enzyme using a novel nontoxic His-tag method based on chelated Fe(III) ions, were tested. Selectivity chain (diols-diesters) and recyclability studies in solvent-free environment were conducted. Results not only revealed a peculiar substrates specificity but also a retention of activity >94 % over 24 h reaction cycle claiming the potentiality of new immobilization strategy. In addition, degrading capabilities of this enzyme against aromatic (PET) and aliphatic biopolyesters (PBS, PHBV, PLA) were investigated. Two glycosylation sites knock out mutants, recombinantly expressed in *Pichia pastoris*, were tested in comparison to wild-type (wt) enzyme. Data claimed that rThc_Cut1 and its mutants hydrolyse aromatic and aliphatic polyester powders at different rates. The best performances were observed against PBS with concentration of released products 10-fold higher than PHBV and PLA. It is worth of note that one mutant was found to be significantly more active on both powder and PBS films than the wt. These results together with the high activity of variants of rThc_Cut1 on PET provide a significant contribution toward enzymatic degradation of polyesters. To make PHA production environmental sustainable and economically competitive, the use of inexpensive substrates was also investigated: waste frying oils (WFOs) were tested as substrates. The commercial value of WFO is depending on several factors and the free fatty acids (FFAs) content strongly decrease the price since it strongly affects the yield of the biodiesel production process. Microbial PHA production alternative to acid pre-treatment of FFAs-rich WFO was explored. The introduction of an upstream microbial fermentation step of *ad hoc* systems achieves the 2-fold purpose of reduction of the FFAs content of the waste and of producing added-value products. Lab-scale results proved the exploitability of the proposed bioprocess both with recombinant and native PHA producing cell factories. In the case of native microorganism, the effect of extracellular lipase in biopolymers production and FFAs reduction was also investigated. Moreover, to re-introduce into the production flow a by-product of biodiesel production, glycerol was tested as C-source for fermentation. This substrate

was applied for boosting PHAs production in above-mentioned bioprocess as well as main C-source for properly designed recombinant *E. coli* strains (*Omni* strains). The investigation of these systems on media supporting production of different PHA co-polymers laid the foundation for the selection and enhancement of enzymes and metabolic background contributing to biopolymers production.

1. Introduction

1.1 Circular Economy and Biorefinery: Bio-based materials

In the 1966 Bouling first laid the foundation of a new way of look at the world describing the open economy of the past, with its seemingly unlimited resources, and contrasts it with the closed economy of the future. He wrote, "I am tempted to call the open economy the 'cowboy economy,' the cowboy being symbolic of the illimitable plains and also associated with reckless, exploitative, romantic, and violent behaviour, which is characteristic of open societies. The closed economy of the future might similarly be called the 'spaceman' economy, in which the earth has become a single spaceship, without unlimited reservoirs of anything, either for extraction or for pollution, and in which, therefore, man must find his place in a cyclical ecological system which is capable of continuous reproduction of material form even though it cannot escape having inputs of energy." (Bouling, 1966) Aim of the author was to warn the reader and the entire society about the risk of the throwaway-mindset (linear consumption behaviour) emerged since the start of industrial revolution which pushed for disposable products with the explicit purpose of being discarded after use (planned obsolesce). The closed economy introduced by the economist has been then named as *Circular Economy* (CE) and its definition has evolved along the time (Stahel, 1982; Yuan et al., 2006; Geng and Doberstein, 2008; Ellen Macarthur Foundation, 2013) until the most recent proposal that is probably also the most comprehensive. The Ellen Macarthur Foundation, in 2013 defined CE as “an industrial economy that is restorative or regenerative by intention and design”. This definition, as fact, considers at the same time both economic and environmental advantages under the notion of regenerative performance requiring high quality circulation of technical nutrients while ensuring the safeguard of the biosphere (Lieder and Rashid, 2016). CE is founded on shifting from the consumption system “make-use-dispose” (cradle-to-grave) of the linear economy (LE) to closed-loop systems (cradle-to-cradle) by applying the “3R” principles: Reduce, Reuse and Recycle of materials and energy (Lindhqvist, 2000). The deterioration of the environment intrinsically linked to LE is completely overcame by the new economic approach. Traditionally, wastes have been considered as something that is not useful and have often to be neglected over the years, while research is progressing towards developing a closed loop approach. As a fact, effluents coming out from a process can be used as feedstocks for a second process, thus achieving maximum treatment efficiency (**Figure 1.1.1**). This new circular flow of products possesses the potentiality to face the issues of resource scarcity, environmental impact, economic benefits and any combination of them that are challenging governments, industries and societies around the world.

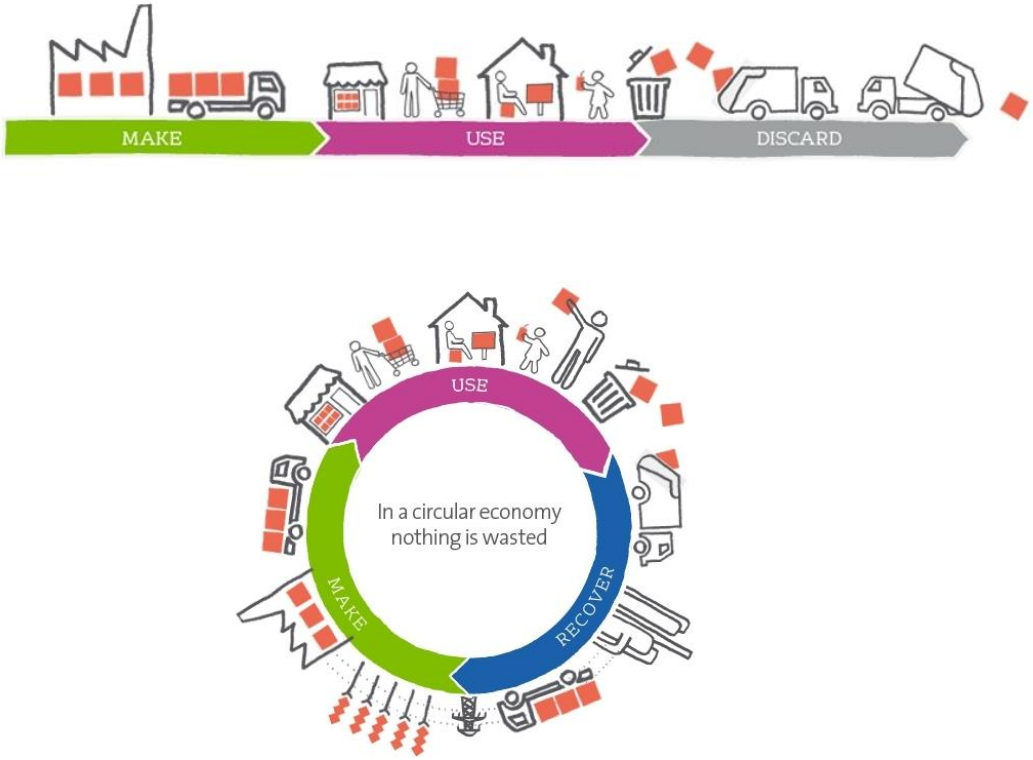

Figure 1.1.1 *Linear Economy vs Circular Economy.*

In frame with the model of CE, in the last years a new class of manufacturing processes is arising: the Biorefinery. A biorefinery is a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass. The biorefinery concept is analogous to that of today's petroleum refineries, which produce multiple fuels and products from oil. By producing multiple products, a biorefinery can take advantage of the differences in biomass components and maximize the value derived from the biomass feedstock.
In 2008, International Energy Agency Bioenergy Task 42 officially defined biorefinery as *the sustainable processing of biomass into a spectrum of marketable products and energy* and developed an unambiguous biorefinery classification system based on a schematic representation of biomass to end-products routes (https://www.iea-bioenergy.task42-biorefineries.com). The classification approach identifies, classifies, and describes the different biorefinery systems according to four main features: platforms (intermediates for connection of different biorefinery systems), products (energy and biobased compounds), feedstocks (energy crops and biomass residues), and conversion processes (biochemical, thermochemical, chemical and mechanical processes) (de Jong and Jungmeier, 2015).

In the context of white biotechnology and biorefinery development, the production of biomaterials represents one of the most promising opportunities to reach not only the environmental sustainability, but also economic and social ones. A CE inspired approach for bioplastics production will assure environmental, social and economic benefits over their whole life cycle, from the supply of raw materials until the final disposal, without comprising the ability of future generations to meet their own needs

(definition of sustainable process adapted from World Commission on Environment and Development's report "Our Common Future", 1987).

Bioplastics

Plastics industry, including plastics producers, plastics converters and the plastics machinery accounted in 2012 for an estimated 1.4 million jobs in the European Union's 27 Member States and had a combined turnover of above 300 billion euro. The plastics industry has benefited from 50 years of growth with a year on year expansion of 8.7% from 1950 to 2012 (http://www.plasticseurope.org). 75% of the plastic demand in EU is composed by mainly five high volume families: polyethylene (PE), polypropylene (PP), polyvinylchloride (PVC), polystyrene (PS) and polyethylene terephthalate (PET). Unfortunately, the use of these plastics constitutes a major environmental problem of the modern society. Together with the massive use of fossil reserves, these synthesized plastics are durable, which makes them resistant to biological degradation. Due to the presence of toxic additives, for example, plasticizers like adipates and phthalate, burning plastic can release toxic pollutants. Further, the manufacturing of chemical industrial processes often creates environmental hazards.

A worldwide concern regarding the research of biodegradable plastic material has been developed during the past years as a remedy towards the harmful effects caused by plastic wastes on the environment. This development has been further pushed by the awareness of having even less support to produce fossil-based plastics and the need of focusing onto next generation of sustainable and renewable sources. The promise of low impact plastics has driven much of the recent work in bio-based materials applications, placed in the general framework of "Green Chemistry", which focuses on minimizing the environmental impact of manufacturing processes through careful management of feedstocks, energy, waste and products.

Today, there is a bioplastic alternative for almost every conventional plastic materials and corresponding application. Depending on the material, bioplastics have the same properties as conventional plastics and offer additional advantages, such as a reduced carbon footprint or additional waste management options such as composting.

Bioplastics industry has come up with numerous innovative technical and material solutions that offer new material properties for improved performances, including enhanced barrier properties, increased material strength, and improved optical properties.

Types of Bioplastics

Bioplastic, chemically termed biopolymers, are polymer produced from renewable feedstock and/or microorganisms.

In recent years several biopolymers have been developed starting from many wellsprings. Possible sources range from different types of biomass including proteins (from animal and vegetal sources which are gaining interest due to their high functionality and excellent properties) (Bugnicourt et al., 2013), lipids and polysaccharides (e.g. starch and cellulose based biopolymers).

Others include bio-based polymers obtained from bio-derived monomers, e.g. from corn, which are then polymerized through standard routes. This is the case for biopolyesters such as polylactic acid (PLA), polyethylene therephtalate (PET) or polyethylene (PE).

Microbial synthesized biopolymers, like polyhydroxyalkanoates (PHAs), have also attracted much attention due to their structural diversity and biodegradability, which makes them extremely desirable substitutes for synthetic plastics.
The most commonly used biopolymers can be classified as follows: bio-based and non-biodegradable plastic, bio-based and biodegradable plastic and fossil-based and biodegradable plastic (**Figure 1.1.2**).

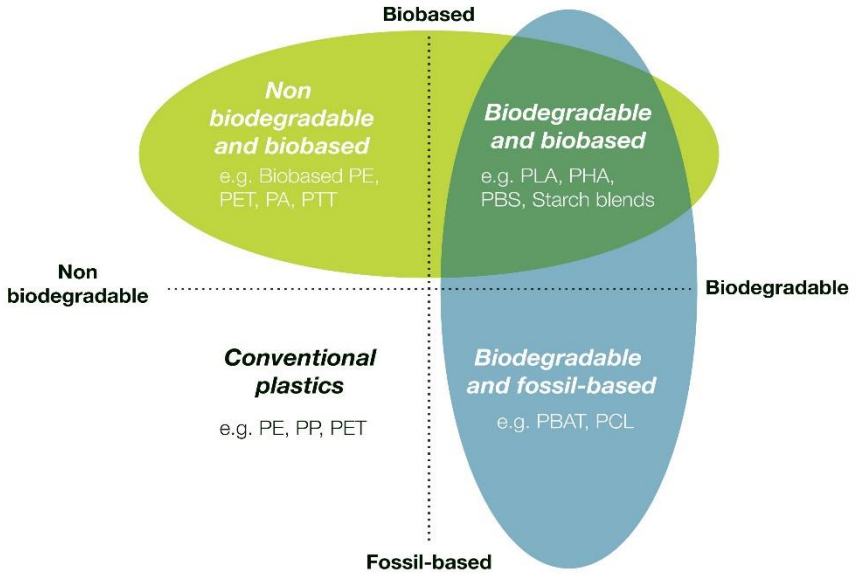

***Figure 1.1.2**: Biopolymers' classification.*

Bio-based and non-biodegradable plastic are polymers made fully, or partly, from natural and renewable resources. These types of non-biodegradable, bio-based plastics are essentially manufactured in the same way as conventional petrol derived plastics but use building blocks derived from biomass instead of fossil fuels. They are designed to be identical to conventional plastic and can be fully recycled with it.
Bio-based and biodegradable plastics (PLA, PBS and PHA) are also made fully, or partly, from biomass but, differently from the ones previously described, are biodegradable, since their composition is based on building blocks which are susceptible to bio-catalysed hydrolysis (PLA requires particular conditions to effectively break down). To complete the classification, plastics that are made from fossil-based resources that can biodegraded and composted also need to be mentioned; however this kind of plastic is not very common. Commercial examples such as polybutylene adipate terephthalate (PBAT), and polycaprolactone (PCL) are available (Elvers et al., 2016).

Applications and market sectors

Although bioplastics are used in an increasing number of markets packaging remains the largest fields of application with almost 40 percent (1.6 million tonnes) of the total bioplastics market in 2016. An increase in the uptake of bioplastic materials in many other sectors was also observed: consumer goods (22 percent, 0.9 million tonnes), automotive and transport sector (14 percent, 0.6 million tonnes) and construction and building sector (13 percent, 0.5 million tonnes).

Economic and social development

Financial investment made into production and marketing has led to a steadily development of new bio-based materials for new applications and products by the newly founded manufactures which sell to an increasing number of conscientious end-users. All these progresses have been possible thanks to the fruitful legal framework conditions providing incentives for the use of bioplastics in several countries worldwide. According to the recent job market analysis conducted by EuropaBio (European Bioplastic, 2016) the nascent bioplastic industry, over the coming decades, not only will contribute to move towards a sustainable future with a reduced environmental impact but it will also unfold an immense economic potential realising a steep employment growth. As fact, a tenfold increase accounted jobs in Europe is forecasted by 2030, starting from the 23,000 of the 2013 statistic reaching up to 300,000 high skilled jobs in European bioplastic (European Bioplastic, 2016).

Dynamic market growth

Currently, bioplastics represent about one percent of the about 320 million tonnes of plastic produced annually. As demand is rising and with more sophisticated biopolymers, applications, and products emerging, the market is growing by about 20 to 100 per cent per year. According to European Bioplastics, global production capacity is predicted to exceed 6 million tonnes in 2021.

More than 75 percent of the bioplastics production capacity worldwide in 2016 was bio-based, durable plastics. As regard to biodegradable polymers, such as PLA, PHA, and starch blends, production capacities are forecasted to rapidly grow from around 0.9 million tonnes in 2016 to almost 1.3 million tonnes in 2021. **PHA production will almost quadruple by 2021 compared to 2016, due to a ramp-up of capacities in Asia and the USA and the start-up of the first PHA plant in Europe.** (European Bioplastic, 2016)

1.2 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are gaining increasing attention in the biodegradable polymer market due to their promising properties such as high biodegradability in different environments, not just in composting plants, and processing versatility. Among biopolymers, these biogenic polyesters have attracted much attention as potential sustainable replacement for fossil fuel-based thermoplastics (Bugnicourt et al., 2014).

PHAs are polyesters of R-hydroxyalkanoic acids, synthesized and stored as intracellular carbon and energy reserve in Gram-negative and Gram-positive bacteria mainly in response to stress conditions (nitrogen, phosphorus, oxygen limitation or pH shifts). Accumulated PHA accounts for 30-50% of the dry weight of most bacterial cells and under starvation conditions they are degraded to maintain cellular energy homeostasis (Sudesh et al., 2000; Lu et al., 2009; Jendrossek, 2009).

On the basis of their monomers' composition, PHAs have been classified into short-chain length (SCL-PHA) and medium chain length (MCL- PHA). Short-chain-length PHAs, containing monomers from 3 to 5 carbons atoms, are the most common polymers, they are stiff, brittle and possess a high degree of crystallinity. Medium chain-length PHAs, containing monomers from 6 to 14 carbons atoms, are flexible polymers with low crystallinity (**Table 1.2.1**).

More than 100 different monomer units have been identified as constituents of PHAs. This creates a possibility for producing different types of biodegradable polymers with an extensive range of properties. Molecular mass of PHA is in the range of 50,000-1,000,000 Da and varies with the PHA producer. A vast majority of microbes synthesize either SCL-PHA containing primarily 3-hydroxybutyrate (3HB) units or MCL-PHA containing 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) as the major monomers (*Tan et al., 2014*). PHB is the most produced and best characterized PHA.

Properties	PHA $_{SCL\ 3-5}$	PHA$_{MCL\ 6-14}$	PP
T_m (°C)	177	61	176
T_g (°C)	2	-36	-10
Crystallinity (%)	70	30	60
Elongation a break (%)	5	300	400
Price (€/Kg)	5*	>1000**	<1

***Table 1.2.1**: Material properties of scl- and mcl- PHAs compared to those of PP. *Price reported by Dietricha (Dietricha et al., 2016), **Price relative to quotation of Polyferm Canada products.*

PHAs biosynthesis

Many bacteria synthesize PHAs as storage compounds forming insoluble cytoplasmic inclusions. Two fundamental steps characterize their production: generation of (R)-hydroxyacylCoAs (RHA-CoAs) and RHA-CoAs' polymerization, forming PHAs. RHA-CoAs are synthetized by a group of enzymes, whose more representatives are: β-ketothiolase, 3-ketoacyl-CoA reductase and enoyl-CoA hydratase; these molecules are then polymerized by a second group of enzymes, called PHAs synthases. PHA synthases play a central role in PHA polymerization in cells. Unlike other biological macromolecules such as proteins and nucleotides, PHA polymerization process does not require the use of a template. PHA synthases polymerize the acyl moiety of the CoA substrate by releasing free CoA and generating long polyester chains that are stored as water-insoluble inclusions of PHA in cells (Tan et al., 2014).

PHA synthases are classified into four groups according to their substrate specificity and subunit composition (Rehm, 2003) (**Table 1.2.2**). Class I synthases are composed of single subunits of PhaC (molecular mass = 60-70 kDa) and their encoding gene (*phaC*) is often found clustered with *phaA* and *phaB*, which encode for β-ketothiolase and NADPH-dependent acetoacetyl-CoA reductase, respectively. Class I, represented by the *Ralstonia eutropha* (*Cupriavidus necator*) enzyme, manly polymerizes short-chain-length monomers (C3-C5), whereas class II, represented by the *Pseudomonas oleovorans* enzyme, polymerizes medium-chain-length monomers (C6-C14). These last ones are also composed of just one type of subunit, PhaC, encoded by two genes (*phaC1* and *phaC2*) that are part of an operon, in which they are separated by *phaZ* gene (intracellular PHA depolymerase). Class III and IV PHA synthases, represented by the *Allochromatium vinosum* and *Bacillus megaterium* enzymes, respectively, consists of two heterosubunits. The catalytic subunit PhaC of these classes (40 kDa) requires the secondary subunits PhaE (40 kDa) and PhaR (20 kDa), respectively, for the full expression of the PhaC's activity (Tsuge et al, 2015). Both class III and class IV synthases prefer to use three to five carbon atoms RHA-CoAs as substrate.

Class	Subunits		Occurrence	Natural Substrates
I	**PhaC**		*Cupravidus necator*	$3HA_{SCL}$-CoA; $4HA_{SCL}$-CoA
II	**PhaC**		*Pseudomonas oleovorans*	$3HA_{MCL}$-CoA
III	**PhaC**	**PhaE**	*Allochromatium vinosum*	$3HA_{SCL}$-CoA; $4HA_{SCL}$-CoA
IV	**PhaC**	**PhaR**	*Bacillus cereus*	$3HA_{SCL}$-CoA

Table 1.2.2: *PHA synthases classification.*

Other enzymes are also involved in PHA synthesis' regulation at transcriptional and translational level and in stabilizing PHA's granules in bacteria, such as PhaR, PhaF and phasins. Many proteins including PHA synthase, PHA depolymerase, granule associated proteins (PhaP) and repressor proteins were reported to be linked on the surface of intracellular PHA granules (Pfeiffer and Jendrossek, 2012).
PHA synthesis and composition depends on a number of factors, including the nature of carbon and nitrogen sources, their concentration ratio in the medium, partial oxygen pressure, the adopted metabolic pathway and others (Bonartsev et al, 2007).
PHA are produced from a wide variety of substrates such as renewable resources (sucrose, starch, cellulose, triacylglycerol), fossil resources (methane, mineral oil, lignite, hard coal), by products (molasses, whey, glycerol), chemicals (propionic acid, 4-hydroxy- butyric acid) and carbon dioxide.
It is possible to classify the selected carbon source, basing on the nature, as "related carbon source" and "unrelated" ones. In the first case, the monomers produced are structurally similar to the given carbon source, whereas in the second case the monomers are not structurally connected to the given carbon source.
There are several biosynthetic pathways for the biosynthesis of PHAs and the most known three are reported in the figure below (**Figure 1.2.1**):

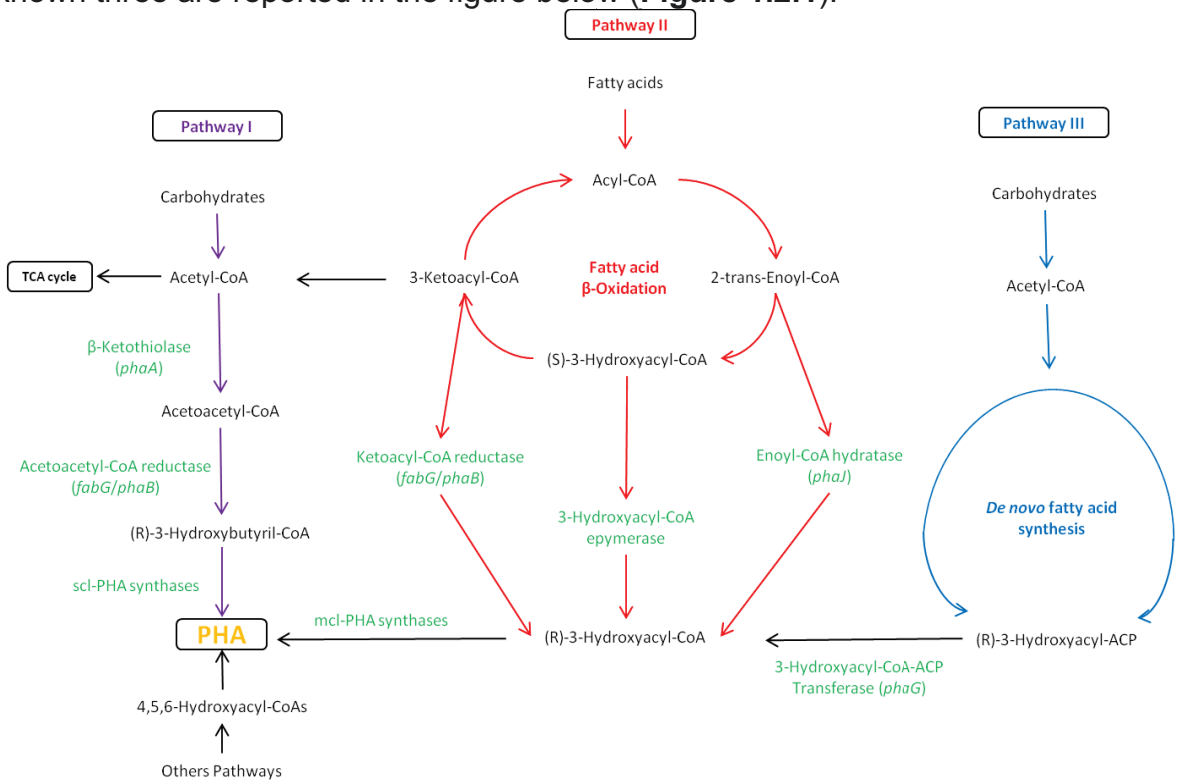

Figure 1.2.1: *PHA biosynthetic pathways:* ***I****) Related carbon source;* ***II****) Fatty acid degradation;* ***III****) Fatty acid biosynthesis.* ***PhaA****:β-ketothiolase;* ***PhaB****:ketoacyl-CoA reductase;* ***PhaG****:transacylase;* ***PhaJ****:enoyl-CoA hydratase;* ***TCA cycle****: tricarboxylic acid cycle. Adapted from Tan et al. 2014.*

1. **Pathway I**: hydroxybutyrate (HB) monomers can be generated from two acetyl-CoA molecules (unrelated carbon source);
2. **Pathway II**: intermediates of fatty acids degradation are channeled from β-oxidation into fatty acid metabolism (related carbon source);
3. **Pathway III**: includes the fatty acid biosynthetic pathway that produces (R)-3-hydroxyacyl intermediates (unrelated carbon source).

PHA production in bacteria

PHA-producing bacteria can be divided into two groups according to culture conditions required for PHA synthesis. The first group requires the limitation of an essential nutrient(s) for the production of PHAs. Bacteria in this group include *C. necator, Rhodopseudomonas palustris* and *Methylobacterium organophilum.* The second group synthesize PHAs alongside its growth in the cultivation medium. Bacteria in this group include *Alcaligenes latus* and recombinant *E. coli* containing the PHA biosynthetic genes (Koller et al., 2017).

Native PHA production

C. necator has been widely studied because of its potential in producing significant amounts of PHB from simple carbon substrates such as glucose, lactic acid and acetic acid (Ryu et al., 1997). Also, olive oil, corn oil and palm oil have been used to produce approximately 80% dry cell weight (dcw) PHB of dry cell mass from the organism (Futui and Doi, 1998). *Methylobacterium organophilum* produces PHA from the cheap carbon source, methanol. However, PHA production from glucose (53% dcw) and sucrose (40% dcw) gave better yield than when methanol (11% dcw) was utilized as carbon source (Kim et al., 1996).

Alcaligenes latus is an organism that produces PHAs using carbon sources such as glucose, molasses and sucrose with good yields. PHA accumulation of approximately 88% dcw (dried cell weight) has been achieved after 8 h nitrogen limitation and 20 h total cultivation time from *A. latus* ATCC 29713, using sucrose as the sole carbon source (Wang and Lee, 1997). *A. latus* requires a temperature of 35°C, which makes P(3HB) production from the organism economic–lowering the demand for cooling during fermentation. Also, PHA production in *A. latus* is growth-associated; however, in condition of nutrient limitation, PHA productivity in *A. latus* can be further enhanced. Optimum biomass and PHA accumulation can also be enhanced by feeding the organism with enriched medium (Anjum et al., 2016).

Among the *Pseudomonas* species, *P. oleovorans* NRRL B-778 has been shown to accumulate mixtures of P(3HB) and MCL-PHAs such as 3-hydroxyoctanoate P(3HO), when fed with mixtures of glucose and octanoic acid, while copolymers with 3-hydroxyvalerate P(3HV) are produced during growth with nonanoic acid (Ashby et al., 2002). It has been observed that, while media containing *n*-alkanoic acids specifically from formate to decanoate as carbon sources, are able to support the growth of *P. oleovorans*, only hexanoate and *n*-alkanoic acids with a number of atom of Carbon higher than six, were able to support PHA accumulation in the organism (Brandl et al., 1988). Generally, *P. putida* efficiently incorporates monomers in the range C8 −C10. Long chain fatty acids such as oleate (C18) are less efficiently utilized because of the need for the *β*-oxidation pathway leading to the production of C8 and C10 monomers that can then be incorporated into the PHA (Fontaine et al. 2017). Polymer yield per cell is often very high when medium-chain fatty acids are employed as sources of carbon, however, medium-chain fatty acids are very expensive and lead to an increase in the production cost.

Recombinant PHA production (E. coli)

E*. coli* has been one of the most favoured recombinant hosts since growth-related PHA production is possible (Leong et al. 2014). Furthermore, the ability to grow fast, to achieve high cell density from several inexpensive carbon sources, e.g. molasses and whey, and easy purification of the polymer from *E. coli*, have contributed to its popularity (Hahn et al., 1995; Fidler et al., 1992). With the vast knowledge of *E. coli* genetics and of its metabolic pathways, *E. coli* is expected to continue to play a significant part in the commercial production of PHAs. Recombinant *E. coli* harbouring the *C. necator* PHA biosynthetic genes were able to accumulate P(3HB) with a yield of 80–90% dcw in fed-batch cultivation while a P(3HB) content of 76% dcw was obtained in a pH-stat fed-batch culture (Kim et al., 1992). Liu and coworkers, obtained a P(3HB) concentration of 80% dcw when recombinant *E. coli* containing the *C. necator* PHA biosynthetic genes was grown on molasses (Liu et al., 1998). Studies have also proven that recombinant *E. coli* with the *Aeromonas hydrophila* biosynthetic genes (*orf1*) can produce terpolymers of P(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3- hydroxyhexanoate) using decanoate and odd-chain fatty acids as carbon sources (Park et al., 2001).

PHA production in mixed or co-culture systems

Mixed or co-culture systems have also been shown to be effective for PHA production (Tanaka et al., 1995; Moralejo-Gárate et al., 2011; Moita et al., 2014). *C. necator* is unable to metabolize sugars, molasses, whey or starchy waste. Consequently, mixed cultures of lactic acid producing bacteria such as *Lactobacillus lactis* (Tanaka et al., 1995)*, Propionibacterium* (Tohyama et al., 2002) and *Lactobacillus delbrueckii* (Patnaik, 2005) and *C. necator* have been used in a single-stage fermentation system.

PHAs' applications

PHAs are promising alternatives polymers to petrochemical plastics for a variety of short life and single-use applications. Interest in the use of PHAs for packaging, medical, agricultural and fisheries applications has recently increased. PHA have been also applied in depolymerized form in fine chemistry and biofuels sectors. There are further applications of PHAs and PHA composites and nanocomposites within the medical and pharmaceutical industries, primarily due to their biodegradability and biocompatibility. Biodegradation experiments of polymer films in marine and soil environments indicated that, in general and depending on the environment, biodegradation rates for unblended polymers are: polyhydroxybutyrate-co-valerate > cellophane> chitosan polycaprolactone. In vivo biocompatibility has been also reviewed in detail (Galego et al., 2000).

Possible PHA application are summarized in **Table 1.2.3**.

Applications	Examples
Packaging industry	All packaging materials that are used for a short period of time, including food utensils, films, daily consumables, electronic appliances et al.
Printing &photographic industry	PHA are polyesters that can be easily stained
Other bulk chemicals	Heat sensitive adhesives, latex, smart gels. PHA non woven matrices can be used to remove facial oils
Block copolymerization	PHA can be changed into PHA diols for block copolymerization with other polymers
Plastic processing	PHA can be used as processing aids for plastic processing
Textile industry	Like nylons, PHA can be processed into fibers
Fine chemical industry	PHA monomers are all chiral R-forms, and can be used as chiral starting materials for the synthesis of antibiotics and other fine chemicals
Medical implant biomaterials	PHA have biodegradability and biocompatibility properties, and can be developed into medical implant materials. PHA can also be turned into drug controlled release matrices
Medical	PHA monomers, especially R3HB, have therapeutic effects on Alzheimer's and Parkinson's diseases, osteoporosis and even memory improvement *et al*.
Healthy food additives	PHA oligomers can be used as food supplements for obtaining ketone bodies
Industrial microbiology	The PHA synthetic operon can be used as a metabolic regulator or resistance enhancer to improve performances of industrial microbial strains
Biofuels or fuel additives	PHA can be hydrolyzed to form hydroxyalkanoate methyl esters that are combustible
Protein purification	PHA granule binding proteins phasin or PhaP are used to purify recombinant proteins
Specific drug delivery	Coexpression of PhaP and specific ligands can help achieve specific targeting to diseased tissues

Table 1.2.3: *Applications of PHA in various fields. Adapted from Chen, 2009.*

1.3 Aim of the thesis

The overall aim of this thesis project was to develop new tools and strategies for biopolymers synthesis, functionalization and degradation. Among biopolyesters, PHAs was selected as target biomaterials to be synthetized and functionalized. In the **Section 2** recombinant cell factories were designed and constructed to achieve the production of desired PHAs (**Chapter 2.1**).
System engineering strategies were applied to improve biopolymer productivity resulting in 6-fold mcl-PHAs yield increase. Biopolymers production was performed in bioreactor and the purified polymer was functionalized. A new enzymatic (lipase) based approach for biopolyesters functionalization was developed and validated for coupling recombinantly produced PHA with dimethylitaconate and polyethylene glycol (**Chapter 2.2**).
Enzymatic approach was also followed to biopolyesters synthesis and degradation. The potentiality of a recombinantly produced cutinases were investigated in synthesis of oligoesters (**Chapter 2.3**) and degradation of aromatic and aliphatic polyesters (**Chapter 2.4**).

With the aim to develop a sustainable biorefinery in the **Section 3** biopolymers production from waste materials was exploited. Waste frying oils were tested as substrate for recombinant (**Chapter 3.2-3**) and native PHA production (**Chapter 3.3**). Analysis of PHAs production in native microorganisms was carried out on waste frying oils with a high free fatty acids content which makes them unsuitable for biodiesel production. Obtained results (biopolymers production and purification of waste oils for biodiesel conversion) supported the technology transfer with the establishment of a society (soon start-up) for industrial validation of designed bioprocess for the valorization of low value lipid substrates in biofuels and biopolymers (**Chapter 3.3**).
In frame of BEETOUT project, new recombinant systems were constructed and preliminary tested for production of PHAs copolymers from crude glycerol derived from biodiesel manufacturing (**Chapter 3.5**).

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2. Novel strategies for synthesis, functionalization and degradation of bio-based materials

Production of medium chain length polyhydroxyalkanoates from waste oils by recombinant Escherichia coli

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Research Article

Production of medium chain length polyhydroxyalkanoates from waste oils by recombinant *Escherichia coli*

Medium chain length polyhydroxyalkanoates (mcl-PHAs) are attractive "green" alternatives to conventional petroleum-based plastics, finding application in various fields. However, their sustainable exploitation is still hampered by the high production costs. In this work, an *Escherichia coli* recombinant system has been designed to allow accumulation of mcl-type polymers through conversion of waste materials, such as spent cooking oils. The system has been engineered with a newly isolated PHAs biosynthetic operon from *Bacillus cereus* 6E/2 and tested for PHAs production on different carbon sources. Results have highlighted the peculiar specificity of the designed *E. coli* system to drive the incorporation of 3-hydroxyhexanoate monomers (up to 99%) in produced PHAs, whatever is the related C-source fed to the growth medium: fatty acids with different length, vegetable oils, or complex waste oils. The work also provides first clues about the role played by *B. cereus* PHA biosynthetic proteins in PHA production process, laying the basis for the development of ad hoc designed cell factories for the synthesis of polymers with defined composition.

Keywords: 3-Hydroxyhexanoate / Biopolymer / Homopolymer / Medium chain length / PHA

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1 Introduction

Polyhydroxyalkanoates (PHAs) are polyesters, synthesized, and stored as intracellular granules by numerous prokaryotes, which have attracted industrial attention as environmentally friendly and biodegradable alternatives to petroleum-based plastics. To date, more than 150 (*R*)-hydroxyalkanoic acids have been identified as monomer constituents of natural PHAs [1]. Two main groups of polymers can be classified depending on the number of carbons in the monomer: short-chain length PHAs (scl-PHAs) having three to five carbon atoms and medium-chain length PHAs (mcl-PHAs) having six to 14 carbon atoms. Differences in repeating unit composition influence the physical properties of PHAs [2]. scl-PHAs show thermoplastic material properties similar to polypropylene, while mcl-PHAs possess elastic material properties similar to rubber [3,4]. Due to their high elasticity and low crystallinity, mcl-PHAs polymers have emerged as suitable materials for novel applications in cosmetics, paint formulations, other coatings, medical devices, and tissue engineering [5].

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Abbreviations: PHA, polyhydroxyalkanoates; **mcl**, medium chain length; **scl**, short chain length; **3HHx**, 3-hydroxyhexanoate; **3HB**, 3-hydroxybutirrate; **WFO**, waste frying oil; **CDW**, cell dry weight

Monomer composition of produced PHAs depends on the nature of the supplied carbon source and on the metabolic pathway fueling precursors for PHAs biosynthesis [6]. A simplified scheme of the main metabolic routes driving PHA biosynthesis and of the key proteins involved is depicted in Fig. 1. Although more than 300 types of microorganisms have been shown to accumulate PHA polymers, only a few of them are effectively exploitable to industrial scale [7]. While scl-PHAs production has already reached pilot and industrial scale, this is not the case for mcl-PHAs.

Efforts to enhance mcl-PHAs production yield from native accumulating bacteria have taken advantage of metabolic engineering strategies acting on pathways responsible for monomers supplying, such as fatty acid biosynthesis and degradation routes. Examples of mcl-PHA homopolymers and/or near homopolymers have been reported by properly designed β-oxidation defective mutants of *Pseudomonas* species, fed with strictly defined related C-sources [8,9]. Analogously, engineered metabolic pathways have been reconstructed in *E. coli* to promote incorporation of mcl-precursors by exogenous feeding of defined fatty acids, resulting in the production of homopolymers [10] and/or mixed copolymers [11]. Due to the high costs related to the supply of required fatty acids, research has been also focused on engineering metabolic pathways in *E. coli* to produce mcl-PHAs from inexpensive unrelated carbon sources, such as glucose or glycerol, potentially obtainable from cellulosic biomass and as byproduct

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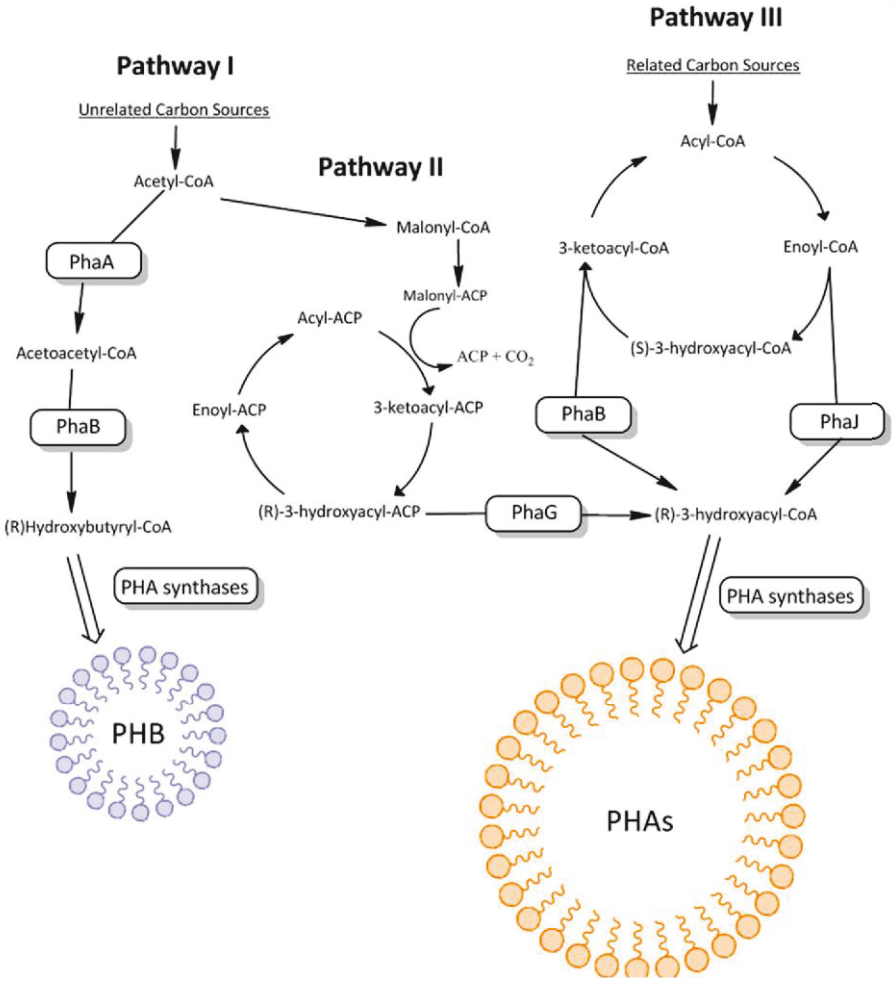

Figure 1. Metabolic pathways for PHA biosynthesis: Pathway I leads to the biosynthesis of PHB polymers by furnishing 3-hydroxybutyryl-CoA precursors from metabolism of unrelated carbon sources; Pathway II and III supply precursors for mcl-PHAs by channeling intermediates from fatty acid synthesis and degradation metabolisms, respectively. The key enzymes responsible for diverting precursors toward polymer biosynthesis are highlighted. PhaA: β-ketothiolase; PhaB: ketoacyl-CoA reductase; PhaC: PHA synthases; PhaG: 3-hydroxyacyl ACP-CoA transacylase; PhaJ: enoyl-CoA hydratase

of biodiesel production, respectively. In these cases, key enzymes of fatty acid biosynthetic pathway [12] or their combination with β-oxidation enzymes and an acyl-ACP thioesterase [13] have been exploited.

In view of process sustainability, the use of waste materials as carbon sources represents a valuable alternative to reduce mcl-PHAs production costs. In particular, waste frying oils (WFO) are a promising source of related carbon source for mcl-PHA bioprocess. However, the heterogeneous and not-reproducible nature of these wastes, together with the complexity of the metabolic routes fueling PHA precursors, make the achievement of a biopolymer with defined composition a challenging goal to be pursued. As a fact, despite the high production levels achieved, 20–40% of cell dry weight (CDW), only polymers with mixed and variable compositions (from C8 to C16) have been produced from different *Pseudomonas* species fed with WFO as related C-sources [14–18].

In this scenario, a strict control of mcl-PHA repeating unit composition could be achieved through a metabolic strategy based on the action of PHA biosynthetic enzymes endowed with peculiar selectivity toward polymer precursors. By introducing such selective enzymes into an *E. coli* metabolic background lacking of any other monomer refurnishing enzymes, precursors refueling will be assured only by the action of the recombinant proteins.

In this work, we aimed at designing a new *E. coli* recombinant system for the production of mcl-PHAs from WFO. For this purpose, we searched for a new source of PHA biosynthetic genes, focusing on those belonging to *Bacillus* species. Native PHAs producers belonging to *Bacillus* species have been characterized for the presence of PHA biosynthetic systems, belonging to the still poorly characterized class IV group [19]. This group of enzymes has been reported to display substrate specificity toward scl-monomers but there are also examples of *Bacilli* producing copolymers with 3-hydroxyhexanoate (3HHx) [20].

In order to support the accumulation of PHAs with low scl-monomers content, we designed a recombinant system in which precursor refueling is assured only through Pathway III, since key enzymes activating Pathways I and II (PhaA and PhaG) (Fig. 1) have not been included in the system.

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2 Materials and methods

All chemicals were purchased from Sigma-Aldrich (Milan, Italy) unless stated otherwise.

2.1 Bacterial strains and growth conditions

Bacillus cereus 6E/2 was provided by University of Naples, Italy, culture collection. *Escherichia coli* Top 10 (Life Technologies, Monza, Italy) was used as host for gene cloning. *Escherichiacoli* BL21 (DE3) (Novagen, Germany) was chosen as host for recombinant protein production. All bacterial strains were routinely grown in Luria-Bertani (LB) broth [21] at 37°C, supplemented with ampicillin (100 μg/mL) if transformed, unless otherwise specified. LB and M9 minimal media were prepared and used as described [21]. A tenfold water-diluted LB preparation, herein defined as LB 10%, was also tested for PHAs production. Terrific broth (TB) composition consisted of 24 g/L Yeast extract (BD Difco, Milan, Italy), 12 g/LBacto Tryptone (BD Difco), 0.8% glycerol, 9.4 g/L K_2HPO_4, and 2.2 g/L KH_2PO_4.

The carbon sources were added to the cultures in the following concentrations: glucose (2% w/v), sodium octanoate (0.2–0.1% w/v), sodium decanoate (0.1–0.05% w/v), sodium dodecanoate (0.05–0.01% w/v). Coconut and corn oils were purchased by local markets and tested at 3 and 6% v/v concentrations. WFO derived from diverse homemade cooking oil preparations and were added to the cultures at 3 and 6% v/v concentrations.

2.2 Extraction of *B. cereus* genomic DNA

A colony of *B. cereus* was inoculated in 4 mL LB and incubated overnight at 37°C under orbital shaking (200 rpm). The cell suspension was then centrifuged for 15 min at 1300 rcf at 4°C. The cellular pellet was suspended in 1.6 mL lysis buffer (9 g/L of glucose, 3 g/L Trizma-base, 15 mM EDTA) in the presence of 50 μg/mL Proteinase K (Euroclone, Milan, Italy). A spatula tip of lysozyme powder was added, the sample mixed by inversion and incubated at 37°C for 10 min. The sample was then incubated for 5 min at 37°C with 30 μL of 20% sarcosyl, and thereafter extracted twice with phenol/chloroform. Genomic DNA was precipitated from the resulting aqueous phase by the addition of three volumes of cooled 96% ethanol and 1 of 10 volume of 3 M sodium acetate pH 5.2 followed by centrifugation at 15 700 rcf for 10 min at 4°C. The resulting DNA pellet was dried at 37°C and suspended in water. Average length of extracted DNA fragments (around 22 Kbps) was verified by migration on agarose gel.

2.3 Amplification of *phaRBC* gene cluster from *B. cereus* 6E/2

A multiple alignment among class IV operon sequences was performed with Clustal W (available at http://www.ebi.ac.uk/). *Bacillus cereus* B4264 (GenBank:CP001176.1); *B. thuringiensis* BMB171 (GenBank:CP001903.1); *B. cereus* ATCC 14579 (GenBank:AE016877.1); *B. thuringiensis serovar kurstaki* str. HD-1 (GenBank:CP004870.1); *B. cereus* G9842 (GenBank:CP001186.1); *B. cereus* E33 L (GenBank:NC006274.1) were the sequences used for the alignment.

Table 1. Oligonucleotide primers used in this study

Primer	Sequence
phaRBCfw	5′-AAAATAATGAATAGGTTGAATTGTTTC-3′
phaRBCrev	5′-TTAATTAGAACGCTCTTCAAGCC-3′
LphaRBCfwNcoI	5′-AACCATGGCGAATAGGTTGAATTGTTTC-3′
phaRBCrevBamHI	5′-TTGGATCCTTAATTAGAACGCTCTTCAA-3′
sphaRBCfwNcoI	5′-AACCATGGTGATTGATCAAAAATTCGATC-3′

Sequences of restriction sites are underlined.

The oligonucleotide primers phaRBCfw and phaRBCrev (Table 1) were designed on the conserved regions at both operon extremities to drive the amplification of the entire PHA biosynthetic operon from *B. cereus* 6E/2 genome, herein defined *phaRBC*$_{Bc}$. Amplification was carried out using GoTaq® DNA polymerase (Promega Italia, Milan, Italy) according to manufacturer's instructions. The amplified DNA fragment *phaRBC*$_{Bc}$ (~2.6 Kbps) was cloned in pGEM®-T Easy Vector (Promega Italia) and sequenced (PRIMM Sequencing Service, Milan, Italy).

Oligonucleotide primer pairs LphaRBCfwNcoI/phaRBCrevBamHI and sphaRBCfwNcoI/phaRBCrevBamHI (Table 1) were used to amplify two forms of *phaRBC*$_{Bc}$, *LphaRBC*$_{Bc}$, and *sphaRBC*$_{Bc}$, respectively, coding for the long and the short version of PhaR$_{Bc}$, by using *B. cereus* genomic DNA preparation as template. Sequences for proper restriction sites were included in primer design for cloning in pET16-b vector (Life Technologies). Amplification was performed using High Fidelity Q5® polymerase (New England Biolabs, Ipswich, Massachusetts, USA) according to manufacturer's instruction.

2.4 Plasmids construction

Plasmids pET16LPHA, pET16SPHA, pET16ΔRPHA, pET16SPHA* (Table 2) were constructed in this study. First two vectors were derived from pET16-b hydrolyzed with *Nco*I and *Bam*HI (Promega Italia) and ligated to properly digested DNA fragments *LphaRBC*$_{Bc}$ and *sphaRBC*$_{Bc}$, respectively. Standard methods were employed for DNA manipulation [21, 22].

Plasmid deleted of *phaR*$_{Bc}$ coding gene (pET16ΔRPHA) was obtained through enzymatic hydrolysis of pET16LPHA by *Xba*I (three *XbaI* target sites are present within *phaR* sequence). The longest fragment (~7.8 Kbps) was purified from agarose gel and let it to ligate.

Plasmid bringing a gene coding for an inactive variant of PhaC$_{Bc}$ (pET16SPHA*) has been obtained through enzymatic digestion of pET16SPHA by *NdeI*, cutting the vector at two different sites. The longest fragment (~7.9 Kbps) has been purified from agarose gel and let it to ligate. The deletion of the region between the two *NdeI* sites shifts the reading frame eliminating two of three residues of the active site of *phaC*$_{Bc}$ coded protein. The BL21(DE3) clone carrying pET16SPHA* has been used as control strain in all the experiment culture conditions. No PHA production has been observed in all the tested culture conditions.

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Table 2. Plasmids and bacterial strains

Plasmid or strain	Relevant characteristics	Source or reference
Plasmids		
pGEM® -T Easy	T-vector for cloning of PCR products	Promega
pET16b	amp^r, $P_{T7\,lac}$, pBR322 origin	Novagen
pGEM® -T Easy-phaRBC$_{Bc}$	pGEM®−T Easy derivative, *Bacillus cereus* 6E/2 *phaRBC*$_{Bc}$	This study
pET16LPHA	pET16b derivative, *B. cereus* 6E/2 *LphaRBC*$_{Bc}$	This study
pET16SPHA	pET16b derivative, *B. cereus* 6E/2 *sphaRBC*$_{Bc}$	This study
pET16ΔRPHA	pET16b derivative, *B. cereus* 6E/2 *phaBC*$_{Bc}$, Δ*phaR*$_{Bc}$	This study
pET16SPHA*	pET16b derivative, B. cereus 6E/2 *sphaRBC**$_{Bc}$	This study
Strains		
B. cereus 6E/2	Wild-type	
Escherichia coli Top10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *galU galK rps*L (StrR) *end*A1 *nup*G	Life Technologies
E. coli BL21(DE3)	F$^-$ *ompT hsdS*$_B$ (r$_B^-$ m$_B^-$) *gal dcm* (DE3)	Novagen
LPha	*E. coli* BL21(DE3), pET16LPHA	This study
sPha	*E. coli* BL21(DE3), pET16SPHA	This study

All cloned PCR products were checked by sequencing (PRIMM Sequencing Service). Recombinant plasmids were transformed in *E. coli* strains using standard transformation methods [21].

2.5 Nile red staining

For routine analysis, a Nile red solution (Sigma-Aldrich) in DMSO was added to the sterilized growth medium to give a final concentration of 0.5 μg dye/mL medium [23]. LB supplemented with 2% glucose was used for testing PHAs production from *B. cereus* 6E/2.

2.6 PHAs production from recombinant *E. coli* strains

For PHAs production, recombinant *E. coli* strains were cultured in 250 mL Erlemeyer flasks containing 60 mL sterile of each tested growth media. Flasks were incubated at 37°C on a rotary shaker at 250 rpm. In the case of cultures carried out in LB with or without 2% glucose, TB or LB 10%, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.5 mM when the OD_{600nm} reached ~0.4. When octanoate, decanoate, dodecanoate, and oils were added as carbon sources, 0.5 mM IPTG induction was performed at a defined time (5 h from the inoculum) due to the interference in optical density measurements caused by the turbidity of these growth media. In all the cases, effective induction of protein expression was verified by performing SDS-PAGE analyses according to standard methods [22].

2.7 Analysis of PHAs production by GC–MS method

For PHAs analysis, cells were harvested by centrifugation (11 120 rcf for 10 min), washed twice with hexane, and lyophilized. PHAs were converted to the corresponding monomer esters by combining 1 mL of chloroform and 1 mL of 15% H_2SO_4 in methanol v/v with 2–5 mg of lyophilized cells in a 6 mL pyrex glass tube [13]. The mixture was heated at 100°C in a heat block for 3 h followed by neutralization with 2 mL of 100 mg/mL $NaHCO_3$ in water. The mixture was vortexed and centrifuged 797 rcf for 10 min and the aqueous layer was removed by aspiration. The organic phase (1 μL) was analyzed using a Agilent Technologies 6850 A gas chromatograph equipped with a mass spectrometer 5973 N (Agilent Technologies Italia, Milan, Italy) and a Zebron ZB-5 Phenomenex, 30 × 0.25 mm id column (He 1 mL/min) (Phenomenex, Bologna, Italy). The injection was done in splitless mode with $T_{injector}$ at 230°C. The temperature program used was as follows: 40°C → 240°C at 20°C/min, 240°C for 10 min. The MS was operated in scanning mode between 40 and 360 *m/z*. The identification of compounds was performed by comparison of mass spectra with standard ones. PHA contents (mg/L) were calculated by using calibration curves, which in turn were obtained by using either 3-hydroxybutyrate or 3HHx (Larodan Fine Chemicals, Sweden) supplemented with benzoic acid as internal standard. PHAs (%) were expressed as the ratio between produced PHAs (mg) and CDW (mg) of lyophilized cell material.

3 Results

3.1 Isolation of a new class IV biosynthetic operon from *B. cereus* 6E/2

PHAs production ability of *B. cereus* 6E/2 has been preliminary verified through Nile Red staining. Specific oligonucleotide primers, have been used to amplify the entire PHA biosynthetic operon from *B. cereus* 6E/2 genomic DNA.

Sequence of the amplified fragment has been determined and analyzed, allowing the identification of three ORFs, putatively coding for enzymes belonging to the PHA biosynthetic pathway, sharing percentages of identity up to 90% with other class IV enzymes [24]: *phaR*$_{Bc}$, coding for PhaR, homologue to *B. cereus* YB4 PhaR (99.4% identity) a protein involved in PHA biosynthesis [25]; *phaB*$_{Bc}$, coding for PhaB, homologue to other 3-ketoacyl-CoA reductases from *Bacillus* species with

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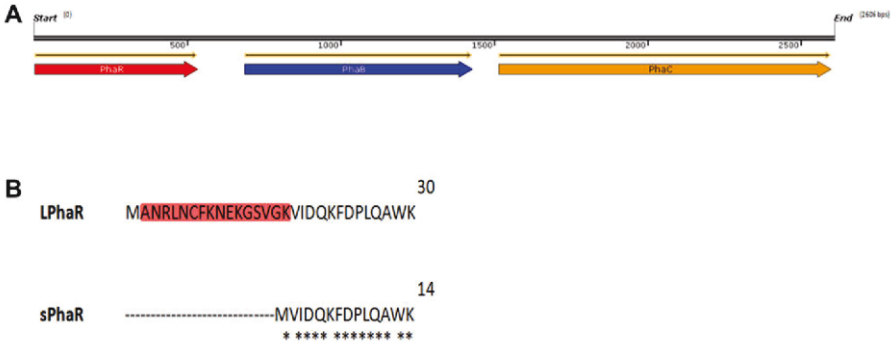

Figure 2. (A) Genetic organization of *Bacillus cereus* 6E/2 *phaRBC* operon; (B) a focus on $LPhaR_{Bc}$ and $sPhaR_{Bc}$ variants highlighting the differences in the 17 aa at N-terminal.

percentage of identity higher than 99%; and $phaC_{Bc}$, coding for PhaC, homologue to class IV PHA synthases, sharing the highest identity (100%) to PhaC from *B. cereus* YB4 (Fig. 2A). Two putative promoter sequences have been identified in the upstream regions of annotated *phaR* genes from *Bacillus* sp. using the Neural Network Promoter Prediction tool for prokaryotes (available at http://www.fruitfly.org/seq_tools/promoter.html). No other putative promoter sequences have been mapped within the entire *B. cereus* 6E/2 amplified sequence, suggesting that the three ORFs are organized in the operon $phaRBC_{Bc.}$ The same genetic organization is shared by other class IV PHA biosynthetic operons and has also been confirmed by a bacterial operon prediction program, FGENESB, (available at http://linux1.softberry.com) selecting *B. cereus* ATCC 10987 as the closest organism.

Two putative translation starts, differing in 17 residues length, have been identified for $PhaR_{Bc}$ coding for a longer ($LPhaR_{Bc}$) and a shorter ($sPhaR_{Bc}$) variant, respectively (Fig. 2B). Since one of the two predicted promoters lays upstream of both putative starting codons and the other is partially overlaid with the $LPhaR_{Bc}$ starting codon, both $PhaR_{Bc}$ variants may be potentially expressed in *B. cereus 6E/2*, from one or both promoters alternatively. Thus, we decided to focus on both versions of the *B. cereus* 6E/2 operons, $LphaRBC_{Bc}$, and $sphaRBC_{Bc}$. Both operons have been cloned in pET16-b and heterologously expressed *E. coli* BL21 cells. The phylogenetic tree (UPGMA method) depicted in Fig. 3, based on aminoacidic sequences of *phaC* coding sequences, shows the distance of $PhaC_{Bc}$ close to the other class IV synthases from *Bacillus* species.

3.2 Analysis of PHAs production from *E. coli* recombinant strains

The performances of the newly isolated $phaRBC_{Bc}$ operons in promoting heterologous PHAs production in *E. coli* have been tested in different growth conditions. The two analyzed *E. coli* recombinant systems, bearing the plasmids pET16LPHA (named *LPha*) and pET16SPHA (named *sPha*), respectively, have been compared in terms of amount of accumulated PHAs and type of incorporated monomer. No PHAs accumulation has been detected in media containing unrelated C-sources (LB, TB, and LB supplemented with 2% glucose) in both recombinant clones. This is in accordance with the designed recombinant system, which does not allow precursors refueling from unrelated carbon sources (Fig. 1). The presence of a PhaG homologue in *E. coli*, FabH, does not seem to supply enough PHA precursors to support PHAs accumulation [26].

When cultures have been carried out in LB supplemented with a related C-source, 0.2% sodium octanoate, PHAs production has been achieved in both recombinant clones. *LPha* has been shown to produce only trace amounts of PHA, while in the case of *sPha*, a PHA content of about 0.3% per CDW (Table 3) has been measured. Interestingly, a significant difference in the pattern of monomer composition has been observed between the two clones: expression of the $LphaRBC_{Bc}$ operon led to the accumulation of a 3-hydroxybutirrate (3HB) containing polymer, while in *sPha*, the major repeating unit in the PHA polymer is 3HHx (99.8 vs. 0.2% 3HB).

A diluted LB preparation (LB 10%) has also been tested in order to promote the utilization of the supplemented C-source (octanoate). An increase in PHA production has been achieved in *sPha* (about 10% per CDW) with almost exclusive incorporation of 3HHx units (about 99.9%). On the other hand, no significant differences have been revealed for biopolymer production in *LPha*.

The recombinant clone bearing pET16ΔRPHA plasmid, deleted of $phaR_{Bc}$ displayed the same behavior of *LPha*, since only trace accumulation of 3HB containing polymer has been detected in the same growth conditions (LB 10% supplemented with 0.2% sodium octanoate).

3.3 PHAs production from different related carbon sources

Further investigations have been focused on *sPha*, in order to verify the effect of different *n*-alkanoates on the monomeric pattern of produced PHAs (Table 3). Sodium octanoate, decanoate, and dodecanoate have been supplied to LB 10% growth medium at two different concentrations, taking into account their toxicity thresholds [27]. PHA accumulation has been observed in all the analyzed conditions, except for the lowest dodecanoate concentration tested, probably due to the very low supply of this carbon source. It is worth to note that, whatever the length of the supplied fatty acid is, the composition of PHAs is characterized almost exclusively by 3HHx monomers with a contribution of 3HB monomers ≤0.1%. No other 3-hydroxyalkanoic acids have been being detected by GC-MS analysis.

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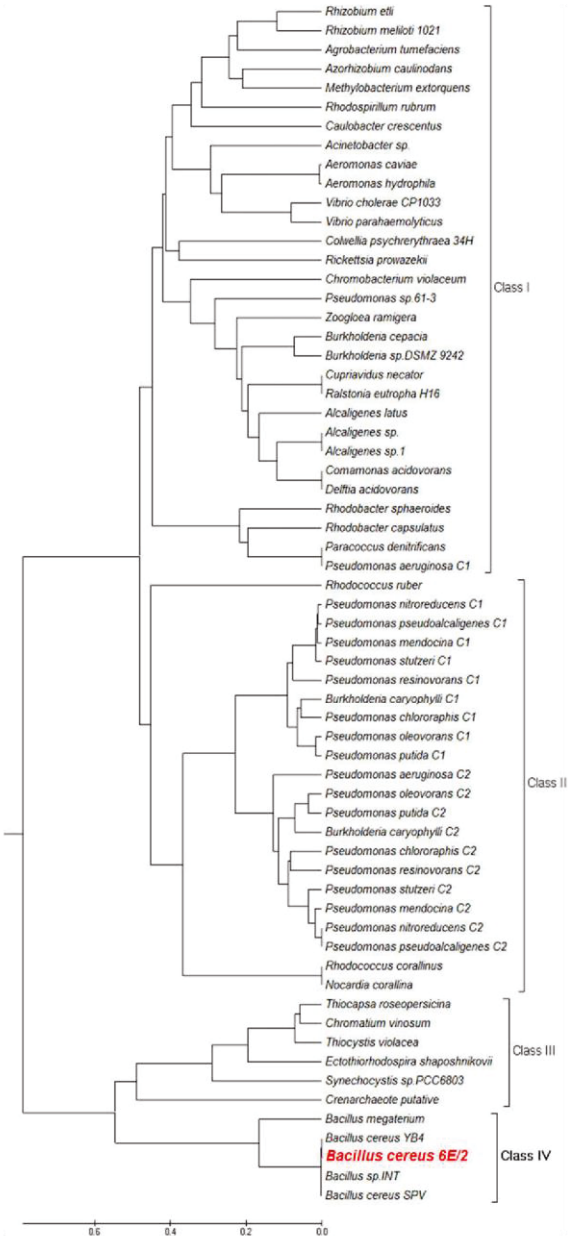

Figure 3. Evolutionary relationships of PhaC amino-acid sequences from 63 taxa. The evolutionary history was inferred using the UPGMA method [40]. The optimal tree with the sum of branch length = 12.03998005 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [41] and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 331 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 [42].

PHA production performances of *sPha* have been also investigated on complex related carbon sources, such as two oils with different mixed fatty acids composition (C8–C20), corn and coconut oils. PHA accumulation has been detected only at the highest oil concentration tested of 6% v/v (about 1% CDW for corn oil). The specificity in driving 3HHx incorporation into PHA polymer has been confirmed also in these cases, with up to 99.5% 3HHx content in the produced PHA, against about 0.5% 3HB. These results indicated that incorporation of 3HHx monomers is a strong peculiar feature of *sPha*, and is independent from the related carbon source added.

3.4 PHAs production from waste materials

The peculiar specificity toward the incorporation of mcl-PHA monomers exhibited by *sPha* when fed with different related C-sources, has encouraged testing of its potentialities in a mcl-PHA production process based on valorization of WFO. For this purpose, PHA accumulating yields have been verified using different preparations of homemade WFO as carbon sources (Table 4).

With the aim to improve PHAs production, two different growth media have been tested: LB 10% and M9, a common minimal defined medium used for PHAs production [26, 28]. A comparison of PHAs production yields in the presence of a defined related C-source (octanoate) with respect to 6% WFO is reported in Table 4. Due to the variability of the different WFO preparations tested, a wide range of PHA production levels has been obtained. In all tested conditions, a PHA polymer mainly composed of 3HHx (about 99.5%) has been obtained. For both octanoate and WFO, M9 has allowed the accumulation of higher biomass levels than LB 10%. In LB 10%, the maximum PHA content accumulated in WFO is halved with respect to octanoate, while in M9, WFO addition determines a higher PHA yield with respect to octanoate. This opposite trend can be explained considering the complex and undefined composition of WFO. These waste carbon sources could provide cofactors or additional nutrients to cells grown in M9 medium, causing a better PHAs accumulation with respect to octanoate. On the other hand, when LB 10% is used, they could prevent/delay the utilization of fatty acids by furnishing alternative/competitive C-sources.

4 Discussion

In this study, the potentiality of a new PHA biosynthetic operon from *B. cereus* 6E/2 in driving heterologous mcl-biopolymer production in *E. coli* has been evaluated. A recombinant system has been properly designed in order to assure precursors refueling only through fatty acid β-oxidation. The study was conducted on two *B. cereus* 6E/2 operon variants, *LphaRBC*$_{Bc}$ and *sphaRBC*$_{Bc}$, identified by in silico analysis of the amplified sequence, which differ in the length of PhaR$_{Bc}$. Results have highlighted the peculiar specificity of the *sPha* to drive the incorporation of 3HHx monomers in produced PHAs whatever is the additional related C-source: fatty acids with different length and vegetable oils.

Plant oils, which are known to produce higher yields of PHA per gram of substrate, have gained much attention as

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Table 3. PHA production in recombinant *Escherichia coli* strains

Strain	Growth medium	Additional carbon source	CDW (mg/L)	PHA (%)	Composition (%)		
					3HB	3HHx	3HA
LPha	LB/TB	/	980.1/4264.2	0	ND	ND	ND
	LB/TB	2% Glucose	1008.2/2986.4	0	ND	ND	ND
	LB	0.2% Octanoate	487.3	trace	100	ND	ND
	LB 10%	0.2% Octanoate	244.9	trace	100	ND	ND
sPha	LB/TB	/	885.2/3998.4	0	ND	ND	ND
	LB/TB	2% Glucose	942.6/2654.8	0	ND	ND	ND
	LB	0.2% Octanoate	380.0	0.3	0.2	99.8	ND
	LB 10%	0.2% Octanoate	156.2	9.9	0.05	99.95	ND
	LB 10%	0.1% Octanoate	128.0	7.0	0.1	99.9	ND
	LB 10%	0.1% Decanoate	185.7	5.7	0.1	99.9	ND
	LB 10%	0.05% Decanoate	334.9	4.9	0.1	99.9	ND
	LB 10%	0.05% Dodecanoate	344.7	6.65	0.05	99.95	ND
	LB 10%	0.01% Dodecanoate	381.5	0	ND	ND	ND
	LB 10%	3% Corn oil	480.7	0	ND	ND	ND
	LB 10%	6% Corn oil	553.6	0.9	0.6	99.4	ND
	LB 10%	3% Coconut oil	307.4	0	ND	ND	ND
	LB 10%	6% Coconut oil	365.5	0.4	0.5	99.5	ND

CDW = cell dry weight; 3HB = 3-hydroxybutyrate; 3HHx = 3-hydroxyhexanoate; ND = not detected; PHA = polyhydroxyalkanoates, TB = terrific broth; LB = Luria-Bertani.
The SD of each series of results was less than ±15%.

Table 4. PHA production *sPha* in the presence of WFO

Strain	Growth medium	Additional carbon source	CDW (mg/L)	PHA (%)	Composition (%)		
					3HB	3HHx	3HA
sPha	LB 10%	0.2% Octanoate	156.2	9.9	0.05	99.95	ND
	LB 10%	6% WFO	214.2– 582.5*	0.1– 4.8*	0.4	99.6	ND
	M9	0.2% Octanoate	205.0	0.25	0.0	100.0	ND
	M9	6% WFO	272.5– 886.2*	0.1– 1.6*	0.3	99.7	ND

ND = not detected; CDW = cell dry weight; 3HB = 3-hydroxybutyrate; 3HHx = 3-hydroxyhexanoate; PHA = polyhydroxyalkanoates; LB = Luria-Bertani; WFO = waste frying oil.
*Reported values refer to a range of CDW or production levels obtained testing different preparations of homemade WFO. The SD of each series of results was less than ±15%.

potential feedstock for PHA production [28]. However, in the recent years, the increasing demand for both cooking and biodiesel production has considerably increased the price of edible oils, making their usage for PHA production no longer considered as a cost-effective strategy [29]. In view of process sustainability the performances of *sPha* have been, therefore, tested on waste carbon sources, such as WFO. This approach would satisfy a twofold purpose: controlling pollution and waste oil disposal management problems, and allowing the valorization of a waste material through its conversion into high-added value products.

Examples of utilization of WFO as low-cost feedstock for biobased material production have been described almost exclusively for native PHAs producers [14–18,30]. Bacterial strains, such as *Cupriavidus necator* [30], *Pseudomonas resinovorans* [31], and *Pseudomonas oleovorans* [32], have been reported to convert oil-containing substrates into PHAs with different compositions. *Cupriavidus necator* metabolic background enabled the conversion of lipid feedstocks mainly in P3HB [33] whereas incorporation of a small fraction of mcl-monomers (up to 6%mol 3HHx) could be achieved using an engineered strain of *C. necator* PHB–4 harboring the PHA synthase gene of *Aeromonas caviae* ($phaC_{Ac}$) [34].

On the other hand, capability of several *Pseudomonas* species in converting WFO into high-added value mcl-PHA copolymers has been reported [14–18]. When used as substrates, lipid wastes have shown complex relation with respect to monomer composition of mcl-PHAs formed [35]. As a consequence, production of tailor-made polymers in *Pseudomonales* species from WFO still demands challenging research.

The ad hoc designed recombinant strain described in this work represents a useful tool to obtain tailor-made mcl-polymers from waste sources. As a fact, our results represent the first example of an *E. coli* based recombinant system potentially able to produce an almost homopolymeric mcl-PHA from WFO. A properly engineered *E. coli* system, blocked in β-oxidation and transformed with PhaJ4 and PhaC1 with broad substrate specificity, for the production of mcl-PHA homopolymer has been described by Tappel et al. [10]. However, in this report, a

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strict control of repeating unit composition has been achieved only by supplying defined fatty acid as substrates [10].

Although considerably influenced by the variability of waste composition, the obtained yield in mcl-PHAs (4.83% CDW) from WFO is not so far from the values reported for recombinant *E. coli* strain transformed with a mcl-PHA synthase, *PhaC1*, from *P. aeruginosa* [36] and fed with cheese whey and palm oil as carbon sources. Moreover, in contrast with the specificity toward incorporation of 3HHx displayed by our recombinant systems, the authors reported only the production of mixed mcl-PHA copolymers with variable relative percentages of monomers from 6 to 12 atoms of carbons.

Reported data have also provided first molecular hints about the functioning of *B. cereus* 6E/2 PHA biosynthetic proteins. Results strongly suggest that the monomeric preference toward 3HHx displayed by *sPha* may be the result of the synergic action of two proteins: $sPhaR_{Bc}$, modulating $PhaC_{Bc}$ activity and selectivity and $PhaB_{Bc}$, channeling 3HHx precursors into the growing polymer. The two $PhaR_{Bc}$ forms, $LPhaR_{Bc}$ and $sPhaR_{Bc}$, may act in a different way during the biosynthetic process. Besides allowing effective polymer accumulation, $sPhaR_{Bc}$ seems to play a role in determining the substrate specificity of class IV synthesis complex, driving the preferential incorporation of 3HHx monomers. It has been reported that PhaR from Bacillus *megaterium* is essential for PHAs accumulation and has been found to be associated to the synthetic granule forming biosynthetic complex with PhaC [19]. The presence of 17 aminoacids, mostly positively charged, at the N-terminal of $LPhaR_{Bc}$ could possibly weaken or even prevent its interaction with the synthase $PhaC_{Bc}$, affecting the functioning of the synthetic complex. Results obtained with the clone carrying $PhaR_{Bc}$ deletion further support this hypothesis. Moreover, PhaR from class I, class II, and class III synthetic operons [37–39] has also been proposed to act as a transcriptional regulator. Although it cannot be ruled out that the two different versions of $PhaR_{Bc}$ may play different roles in the overall PHA metabolism when expressed in *B. cereus*, results described in this work point out, for the first time, the importance of PhaR in determining activity and substrate specificity of class IV synthetic complex in a heterologous PHAs production system.

PHA precursors fueling from Pathway III (Fig. 1) is another important aspect to be considered [12] to explain the obtained results. In this regard, PhaB plays a crucial role in subtracting β-ketoacyl-CoA precursors to the β-oxidation cycle by channelling them toward incorporation into the growing polymer. A higher affinity of $PhaB_{Bc}$ toward C6 monomers may explain the observed PHAs composition. Incorporation of low percentages of 3HB in *sPha* probably derives from further oxidation of the portion of 3-ketohexanoyl-CoA molecules that are not reduced by $PhaB_{Bc}$.

Further insights into the role played by *B. cereus* PHA biosynthetic proteins in PHA production process will allow to identify the molecular targets determining monomer specificity, laying the foundations for the development of ad hoc designed engineered systems for the synthesis of polymers with defined composition.

In conclusion, our results have provided a "proof of concept" of the potential exploitation of an *E. coli* recombinant system based on *B. cereus* 6E/2 PHA biosynthetic operon for valorization of WFO into valuable products. The high specificity toward incorporation of 3HHx monomers has allowed the synthesis of a biopolymer with "controlled" monomer content even when waste materials have been used as carbon sources.

Of course, optimization of polymer production yield and its deep characterization will be required for effective exploitation of the developed system. In this regard, proper genetic modification of strain background, designing of stable gene chromosomal integrations and of cost competitive expression systems are the next necessary steps to be undertaken. Not least, analysis of WFO composition and/or the use of wastes from big fast-food chains (providing oils with almost constant content) in combination with different minimal media, will be helpful to define the optimal conditions for PHA production.

Practical application

This research article describes the potential use of *E. coli* recombinant systems, expressing a newly isolated class IV polyhydroxyalkanoates (PHAs) biosynthetic operon from *B. cereus* 6E/2, in a sustainable process for the production of a medium chain length (mcl) PHA polymer. The work provides a proof-of-concept analysis of mcl-polymer accumulation, with preferential incorporation of 3-hydroxyhexanoate (3HHx) moieties whatever is the related C-source fed to the recombinant strain:fatty acids with different length, vegetable oils, or complex waste oils. The work also sheds light on the enzymes responsible for the observed selectivity toward 3HHx monomers, suggesting new targets for the designing of tailored engineered systems for the synthesis of polymers with desired composition.

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2. Novel strategies for synthesis, functionalization and degradation of bio-based materials

Enzymatic production of clickable and PEGylated recombinant polyhydroxyalkanoates

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Enzymatic production of clickable and PEGylated recombinant polyhydroxyalkanoates†

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Polyhydroxyalkanoates (PHAs) are microbial polyesters attracting great attention while further functionalisation could widen their applicability. Various *Escherichia coli* based production systems using the PHA biosynthetic operon from *Bacillus cereus* 6E/2 were designed to enhance the accumulation of PHAs with high medium chain length moieties. Media optimization and system engineering were applied, yielding the production of PHAs up to 260 mg L^{-1}. Polymer characterization revealed a low grade of crystallinity and remarkable hydrophobic features. For further functionalization, a novel enzyme based strategy was developed. Lipase B from *Candida antarctica* (CaLB) was used to catalyze the terminal coupling of PHAs with: (i) dimethyl itaconate (DMI) in order to introduce reactive side chain vinyl moieties for the easy coupling of functional molecules and/or (ii) biocompatible polyethylene glycol (PEG) to tune polymer hydrophilicity. The functionalized DMI–PHA, PEG–PHA and PEG–DMI–PHA polymers obtained and characterised by NMR, GPC, FT-IR, and WCA in this study open new perspectives on the use of PHAs as biodegradable and biocompatible materials of choice for biomedical applications.

Introduction

PHAs are microbial polyesters produced as intracellular energy-reserve granules by a large variety of microorganisms. They are degraded by many bacteria and fungi under aerobic conditions or to methane under anaerobic conditions.[1] Depending on the total number of carbon atoms of the hydroxyalkanoic acid building block, PHAs can be classified as either a short-chain length PHA (scl-PHA; 3–5 carbon atoms), a medium-chain length PHA (mcl-PHA; 6–14 carbon atoms), or a long-chain length PHA (lcl-PHA; >15 carbon atoms).[2] This classic PHA classification, however, does not take into account the effect of crystallinity, which can play a critical role in further processing.[3,4] According to Abe *et al.*, on the basis of their crystallization behaviour, the PHAs should be classified as scl-PHAs when built from C ≤ 6 monomers and mcl-PHAs when built from C ≥ 7 monomers.[5] Thanks to their biodegradability and biocompatibility, the PHAs are considered an environmentally friendly alternative to conventional petroleum-based plastics, finding applications in various sectors (food packaging, paint, printing and photographic materials, nutritional supplement, fine chemicals, *etc.*) with high under-exploited potential in the medical field.[6] However, the most common and easily producible PHA, poly 3-hydroxy butyrate (PHB), is unsuitable for such applications due to its high toughness and brittleness. Moreover, due to its high crystallinity, the PHB is almost recalcitrant to degradation in the body when compared to other polyesters such as PLA and PGA.[7,8] Regarding PHA co-polymers, their lower crystallinity compared with that of PHB results in a faster degradation rate. However, as reported by Yang *et al.* for various PHB-*co*-PHHx,[9] a strict correlation between the co-monomer content and degradation rates has not been found.[10] Common PHA biodegradation products including oligomers and monomers are also not toxic to cells and tissues and some of them have even been studied for their pharmaceutical applications.[11] Due to this feature and other positive properties (biocompatibility, capability to support the growth of various cells and tissues, no immunological response, and adjustable mechanical properties),[11] a number of PHA-based materials have been widely exploited in the biomedical field with particular focus on PHBV and PHBHHx co-polymers.[12,13]

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Attempts to obtain PHA-based biopolymers with target properties and an increased rate of degradation have been mainly focused on further processing in order to obtain low crystalline polyesters, such as blending with more hydrophilic polymers or with other low molecular weight additives, to improve the water penetration properties.[14] PHAs containing functional groups are attractive for applications in the biomedical field allowing further chemical modification.[13] PHA functionalization with various functional groups and a combination with other bio-polymers by chemical reactions and biosynthetic *in vivo* methods has been exhaustively summarized in various recent reviews.[15,16] Biosynthetic approaches aimed at the *in vivo* incorporation of PHA monomers with specific functional groups remain applicable only to a small class of microorganisms fed on *ad hoc* substrates under precise growth conditions.[17,18] The chemical modification of polyesters, in contrast, represents a polymer-independent strategy for PHA functionalization with side-chain groups that can be further tailored to obtain derivatized polymers such as PHA-based graft/block copolymers or to allow crosslinking reactions among the linear chains.[19,20]

In this frame, poly(ethylene glycol) (PEG) is a biocompatible polymer that has been widely combined with a PHA to manipulate its mechanical and physical properties, *i.e.* intrinsic hydrophobicity and the degradation rate of the designed biomaterial. For instance, bulk polymerized PHBHHx/PEG poly (ether ester urethane) block copolymers have showed the enhanced tissue compatibility of the copolymer films *in vivo* and superior physicochemical properties.[21,22]

Also in the case of PEG, *in vivo* approaches (bioPEGylation) and various coupling reactions have been applied to obtain different amphiphilic PHA derivatives. The former approach, avoiding the classic synthetic route demanding the use of metal catalysts and organic solvents, seems to be more attractive in terms of sustainability. However, the products of bioPEGylation are strongly affected by microbial species, the conditions of cultivation and the molecular weight of utilized PEG.[20] Regarding the chemical approach, amphiphilic copolymers have been obtained from telechelic PHAs following several strategies: (i) Sn(II) 2-ethylhexanoate or (ii) dicyclohexylcarbodiimide and 4-dimethylaminopyridine catalysed block co-polymerizations,[15,23] (iii) PHA/PEG poly(ether ester urethane) synthesis by hexamethylene diisocyanate coupling,[24] (iv) atom transfer radical polymerization on Br–PHA–Br as a macroinitiator,[25] (v) "click" coupling reaction of alkyne-terminated PHAs and azide-terminated PEG[26] and (vi) etherification, UV radiation and thiol–ene chemistry with various PEG derivatives.[27]

In this work, the targeted modification of a recombinantly produced isotactic biopolyester was achieved through an enzymatic approach. Although successfully applied for the synthesis of copolyesters containing a low-medium M_w telechelic PHA,[28–30] to the best of our knowledge only few examples of enzymatic covalent functionalisation of PHA-diols have been reported. Here, lipase B from *C. antarctica* (CaLB) was applied to catalyze the coupling of the PHA with the unsaturated dimethyl itaconate and with the hydrophilic biocompatible polyethylene glycol. A previously characterized *E. coli* system based on the *B. cereus* PHA biosynthetic operon (*LipoA*), able to convert fatty acids from different sources into mcl-PHAs,[31] was engineered and together with media optimization led to improved polymer yields. The obtained functionalized polymers open new perspectives on the application of PHAs based on the simple coupling of bioactive compounds assuring the integrity and the thermal stability of the telechelic polymer of interest.

Results and discussion

PHA production optimization

Starting from the *E. coli LipoA* system, two approaches were pursued to improve the polymer production yield: (i) media optimization and (ii) system engineering.

Media optimization. An optimal cultivation medium for PHA production was formulated balancing its main components: the "basal medium", sustaining microbial growth, and the lipid carbon sources (sodium octanoate), driving the accumulation of mcl-PHA ("additional C-source") (Table 1).

When 0.1% sodium octanoate was added to different basal media (MM, MR_1, MR_2 and M9* supplemented with 0.1–0.4% YE), a diverse amount of biomass was obtained (trials 1–6). MR_1 and MR_2 did not efficiently support microbial growth (DCW < 100 mg L^{-1}), whereas a lower biomass accumulation was achieved in MM (trial 1) with respect to M9* media (trials 4–6). On the other hand, a higher PHA % was achieved in MM when compared to M9Y1, M9Y2 and M9Y4 media. Comparing the effect of the YE concentration in trials 4–6, the highest PHA accumulation (mg L^{-1}) was achieved at 0.2%, whereas a further increase to 0.4% caused a drop in the biopolymer yield. This latter result could be explained by the occurrence of catabolite repression phenomena[32] impairing the utilization of the additional C-source in the presence of other preferred ones. PHA production was then tested in MM and M9Y2 media by increasing the sodium octanoate concentration to 0.2% (trials 7 and 8). It is worth noting that while the increase of sodium octanoate had no significant effect on

Table 1 PHA production in the *LipoA* recombinant strain. CDW, cell dry weight; NA, not analyzed (C.D.W. lower than 100 mg L^{-1}). Data reported at 48 h after induction. The standard deviation of each series of results was less than ±15%

	Medium	Sodium octanoate [%]	C.D.W. [mg L^{-1}]	PHA [%]	PHA amount [mg L^{-1}]
1	MM	0.10	128.0	7.0	9.0
2	MR1	0.10	NA	NA	NA
3	MR2	0.10	NA	NA	NA
4	M9Y1	0.10	330.6	0.0	0.0
5	M9Y2	0.10	514.2	2.0	10.3
6	M9Y4	0.10	612.9	0.2	1.2
7	MM	0.20	156.2	9.9	15.5
8	M9Y2	0.20	216.4	0.0	0.0

the biomass concentration in MM medium, the microbial growth in M9Y2 medium was considerably impaired (from 514.2 to 216.4 mg L^{-1}). A lower amount of biomass in condition 8 is probably caused by the toxic effect of the increased concentration of sodium octanoate. As reported by Hiroe *et al.*,[33] this effect could not only impair the microbial growth but also affect the metabolism linked to the PHA, abolishing biopolymer production.

A two-stage cultivation approach, similar to that previously described by Taguchi *et al.* for *E. coli* recombinant mcl-PHA production,[34] was also applied. In the first phase of growth in rich medium, biomass accumulation was achieved. Cells were then switched to a minimal medium supplied with the additional C-source in order to promote polymer production. This approach, however, appears unsuitable for the *LipoA* system since a PHA production lower than 3 mg L^{-1} was observed (C.D.W. 1.437 g L^{-1}).

Two better conditions for polymer production were selected according to the "Highest Intracellular Polymer Percentage, HIP" (7) and the "Highest Yield of Biomass, HYB" (5). It was supposed that these two different scenarios (few cells with high polymer accumulation and many cells with a low polymer content) should lead to different PHAs.

System engineering. Both HIP and HYB were applied to new *E. coli* strains designed to boost PHA production: *LipoB* and *LipoC*. Two new PHA producing systems were developed taking into account (i) the promoter driving the bio-synthetic operon and (ii) the "host metabolic background". The effect of the promoter was tested by changing the promoter sequence driving the expression of the *sphaRBC* operon (strain *LipoB*). On the other hand, the influence of the host metabolic background was investigated, by choosing LS5218 as the *E. coli* strain due to the higher tolerance to fatty acids (strain *LipoC*). The promoter substitution resulted in a change of the pattern of the recombinantly expressed proteins. When the recombinant expression is under the control of the T7 promoter (*LipoA*), it was possible to observe three different intense bands with increasing molecular weight corresponding to those of PhaR, PhaB and PhaC, respectively. It is worth noting that the three bands appeared with the same relative intensity under all tested conditions. In contrast, for *LipoB* and *LipoC*, only the β-ketoreductase PhaB is clearly noticeable in the cell extract. The proteins of the synthetic complex PhaR–PhaC showed only weak SDS-PAGE bands (Fig. S1†). However, the solubilisation of disrupted cells in Triton 80 containing buffer resulted in three overexpression bands with the same intensity as for *LipoA*. Although specific transcriptomic studies would be requested to completely understand the effect of different promoters, the different SDS-PAGE behaviour of the two systems can be rationalised taking into account the possibility of the interaction of the synthetic complex proteins (PhaR–PhaC) with the produced intracellular polymer.

The PHA production abilities of both *LipoB* and *LipoC* were compared to those of *LipoA* under the conditions HIP and HYB selected above (Table 2).

Table 2 PHA production in constructed recombinant systems (rSystems). CDW, cell dry weight. HIP (highest intracellular percentage), MM with 0.2% sodium octanoate; HYB (highest yield of biomass), M9Y2 with 0.1% sodium octanoate. The standard deviation of each series of results was less than ±15%

rSystem	Growing conditions	C.D.W. [mg L^{-1}]	PHA [%]	PHA amount [mg L^{-1}]
LipoA	HIP	156.2	9.9	15.5
	HYB	514.2	2.0	10.3
LipoB	HIP	197.2	23.2	45.7
	HYB	817.1	11.4	93.1
LipoC	HIP	796.4	0.1	0.7
	HYB	1437.0	0.3	4.7

Both systems allowed a higher biomass yield under all tested conditions if compared with *LipoA*. As expected, *LipoC* appeared to better tolerate the high concentrations of fatty acids; however, its productivity was lower than that of *LipoA*. Even when sodium octanoate was supplied up to 6 g L^{-1}, in *LipoC* an increase in the biopolymer yield was not observed (0.5 mg L^{-1} for M9Y2). The obtained results, in accordance with the data recently reported by Kihara *et al.*,[35] revealed the inadequacy of the *E. coli* LS5218 strain for recombinant PHA production based on the class IV biosynthetic operon *phaRBC*. Interestingly, *LipoB* was able to accumulate up to a 6-fold higher amount of PHA per litre than *LipoA*. The strong increase of yield observed in *LipoB* pointed out a crucial role of the promoter in influencing biopolymer accumulation in the BL21 strain. A deeper understanding of the relationships between the expression level of the PHA biosynthetic proteins and the properties of the produced polymers will be the object of future investigation.

Bioreactor scale-up and polymer characterization

PHA production in the *LipoB* system was scaled-up in a 3-L bioreactor under both tested conditions. Under the HIP condition, the scaling up did not affect either the yield of biomass or the intracellular polymer percentage. In contrast, under the HYB condition, the scaling up resulted in a higher yield of biomass (1424 mg L^{-1}) as well as in an increase of the intracellular polymer percentage (18.4%) achieving a PHA yield over than 250 mg L^{-1}.

Polymers were Soxhlet-extracted using chloroform and characterized for their molecular weight distribution (*via* gel permeation analysis) and thermal stability (thermogravimetric analysis). The composition of polymers was analysed by using GC-MS on lyophilized cells and by the ^{1}H-NMR analysis of purified products. Both techniques revealed a molar ratio between the 3HB and 3HHx monomers of 3 : 2 with a variance lower than 10%.

The GPC analysis in $CHCl_3$ of both produced PHAs resulted in an asymmetric peak which was arbitrarily split into 2 biopolymer families by the introduction of an M_w threshold (Table 3 and Fig. S2† for chromatograms).

Interestingly, the most productive condition (HYB) leads to an accumulation of PHA with lower MW and lower intrinsic

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Table 3 GPC results of polymers analysed in $CHCl_3$

	Peak 1			Peak 2		
Sample	M_n [10^3 g mol^{-1}]	M_w [10^3 g mol^{-1}]	IV [dl g^{-1}]	M_n [10^3 g mol^{-1}]	M_w [10^3 g mol^{-1}]	IV [dl g^{-1}]
LipoB-HYB	190	330	0.9	87	97	0.6
LipoB-HIP	377	464	2.2	189	193	0.99

viscosity characterized by a temperature of degradation higher than the polymer produced under the HIP condition (290 *vs.* 273 °C). These diversities supported the hypothesis that the two different producing scenarios of HYB and HIP stimulate the production of biopolymers with different properties. Due to the 4-fold higher productivity than HIP, the polymer produced under the HYB condition was selected for the derivation study reported in this paper. Further investigation into the effect of the promoter and the growing conditions on the biopolymer properties is currently ongoing.

Film casting and characterization

A direct solvent casting was not applicable to the produced PHA due to its sticky properties. Indeed, using this technique only non-homogeneous films were obtained despite the array of PHA concentrations (10–50 mg mL^{-1}) and the organic media (chloroform, toluene, acetone) that were tested. Moreover, the obtained films resulted to be difficult to handle and to be characterized because they hardly stuck on the glass support. PHA films were therefore prepared by drop casting from a toluene solution on a water surface as previously described by Pappalardo *et al.*[36] This technique allowed the preparation of homogeneous floating films that were easily recoverable from the aqueous layer (Fig. S3†). FT-Raman analysis revealed the characteristic bands of amorphous PHA copolymers.[37] In particular, the C=O stretching was observed at 1737 cm^{-1}, while for crystalline polymers it is normally centred at 1725 cm^{-1}. Moreover, several peculiar bands of the amorphous state were detected: δCH_2 and $\delta_{as}CH_3$ at 1451 cm^{-1}; $\nu_{as}CH_3$ at 2988 cm^{-1}; and the unassigned vibration at 1306 cm^{-1}. The ATR-FT-IR spectrum (1650–650 cm^{-1} range) in Fig. 1 further proved the amorphous state of the produced mcl-PHA films in accordance with what was previously reported for the amorphous PHBV.[37]

The changes of the crystallinity degree were analysed for films aged for 7 days from casting at 21 °C and after 24 h at 105 °C. It is well known that aged as well as heat-treated PHA films present a higher level of crystallinity than just-prepared films.[37,38] The crystallinity index (CI) was defined as the ratio between the relative intensity of the bands at 1382 cm^{-1} (independent of crystallinity) and at 1174 cm^{-1} (increases for an amorphous state). The CI measured for just-prepared films was 0.28 while for the aged and heat-treated samples it was 0.30 and 0.31, respectively. The unaltered CI value represents a proof of the high amorphous state of the produced biopolymers. This hypothesis was further supported by the absence of the melting transition in the DSC in the range of −40 to

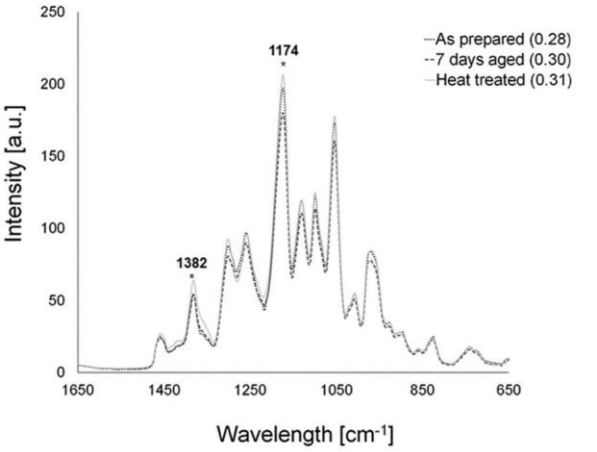

Fig. 1 ATR-FT-IR of a PHBHHx film. The film as prepared, black small dots line; the film aged for 7 days at 21 °C, big dots line; the film heated at 105 °C for 24 h, grey line. In brackets, the crystallinity index of each sample is given.

200 °C. In the DSC experiments only the first heating revealed significant values, whereas the second heating run didn't show any melting endotherms. Once proved that no substantial changes in crystallinity occurred, the film stored at 21 °C under vacuum was selected for the study of surface properties. Water contact angle (WCA) analysis (Fig. S4†) revealed the remarkable hydrophobic properties of the film with an angle of 92 ± 2° that was stable over 10 s. The observed values were in line with the ones reported for PHB, PHBV[39] and PHBHHx.[12,39] The study of surface energy was also approached; however, the marked oil-adsorption of the produced mcl-PHA hampered the measurements, as previously reported by Sudesh *et al.* (Fig. S5†).[40]

PHA functionalization

PHA–DMI. Among the several applications of PHAs, the development of new materials for tissue engineering and drug delivery is gaining attention.[41,42] For these applications, a grafting step with a bio-active compound is often requested and therefore the presence of an easily attachable side chain group (such as a vinyl group) is desirable. In the last few years, itaconic acid (IA) was exploited in combination with several polymers and bio-polymers *via* both chemical and enzymatic approaches.[28,43] In this work, the enzymatic coupling of dimethyl itaconate (DMI) with PHA was studied. In order to

ensure efficient mass transfer, the reactions were performed using toluene, since the thin-film strategy previously described by Pellis *et al.* was not applicable due to the high melting temperature of the used PHA.[28] The organic media were chosen according to reagent solubility and the reported activity of the selected immobilized lipase. Two different PHA–DMI weight/weight ratios were tested: 1 : 0.1 and 1 : 0.4 (PHA–DMI1 and PHA–DMI4). The clear changes of the ^{1}H NMR signals of DMI proved the coupling efficacy (shift of methylene from 3.34 to 3.38–3.42 ppm, intensity reduction of methoxy and a shift from 3.69 and 3.76 ppm to 3.70–3.73 and 3.80–3.83 ppm, and shift of vinyl from 5.71 and 6.32 ppm to 5.81 and 6.39 ppm) (Fig. 2). The relative integration of the NMR peaks of PHA coupled DMI residues was used to follow the PHA esterification grade under the tested conditions. Since no remarkable differences were observed, even at the lowest amount of dimethyl ester, all available hydroxyl end groups of the PHA were saturated under the PHA–DMI1 condition.

Consequently, this condition was selected for further investigations. The GPC analysis of purified PHA–DMI1 revealed the presence of "PHA dimeric species" resulting due to the linkage of two PHA chains by a molecule of DMI as shown in Fig. 3. The relative reactivity of the two different carbonyl groups of DMI in the end-capped PHA was determined from the ^{1}H-NMR spectrum presented in Fig. 2 (for the enlarged product's structures, please see Fig. S6†). The calculated 3 : 1 ratio was in complete accordance with what was reported previously.[44]

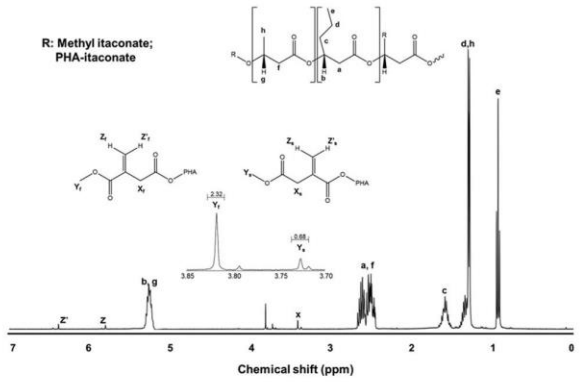

Fig. 2 Full and enlarged ^{1}H-NMR spectrum of PHA–DMI1.

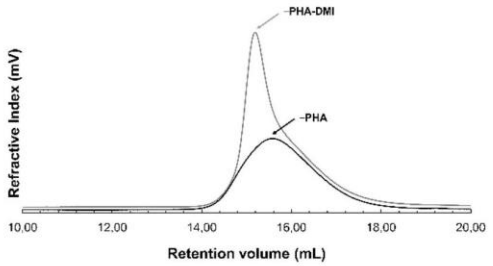

Fig. 3 GPC analysis of PHA (black line) and PHA–DMI1 (grey line).

Furthermore, the PHA–DMI1 was film casted in order to investigate the changes of the materials' surface hydrophilicity. The DMI coupled PHA showed a comparable WCA (around 90°) with respect to the un-reacted PHA, being classifiable as hydrophobic (Fig. S4,† grey line).[41,42]

Thus, the lipase-catalysed transesterification reaction proved to be an effective strategy to add extra unsaturated groups to the PHA, useful for subsequent click-derivatization without altering the surface properties of a polymeric film.

PHA–PEG. Once the efficacy of the enzymatic approach for the transesterification of PHA hydroxylic end groups was proven with a small molecule (DMI), the same approach was adopted to catalyse the reaction at the PHA carbonyl end group with the high molecular weight biocompatible PEG (4000 Da). In order to force the transesterification at the PHA carbonyl end, the reaction was performed in excess of the polyether (PHA : PEG4000 1 : 3 w/w ratio) using 10% of the immobilised catalyst. After the standard work-up, the reaction was vacuum dried and purified in order to remove most of the unreacted PEG4000. The final product was analysed by using ^{1}H NMR. Although the spectrum still revealed the presence of some PEG4000 moieties due to their incomplete purification, clear evidence of coupling was observable in the PEG4000 pattern of signals. As a fact, in agreement with the findings by Chen *et al.*, the signal at 3.72 ppm, assigned to the hydroxyl end groups of PEG4000, strongly decreased after the reaction with PHAs.[45] Residual not-esterified PEG4000 molecules contributed to the leftover signal as evident from Fig. S7.† The coupling was further confirmed by GPC analysis which revealed the presence of a polymer with a higher M_n (61.196 × 10^3 g mol^{-1}) when compared to the unreacted PHA (50.188 × 10^3 g mol^{-1}) (Table 4).

PHA–PEG copolymers have gained high significance for bio-medical applications in the past few years and several strategies have been applied for their synthesis. This work represents the first example of lipase catalysed PHA–PEG4000 coupling. The resulting PHA–PEG copolymer was able to form a stable suspension in a hydrophilic environment (water : acetone mixture 3 : 2) (Fig. 4).

In contrast with what was reported by Loh *et al.* for an amphiphilic thermosensitive PHA–PEG derivative poly(ether ester urethane),[46] the turbid suspension of the obtained PHA–PEG4000 copolymer did not show morphological changes in the −20 to 70 °C thermal range.

Table 4 GPC results of polymers analysed in THF

	M_n	DI	M_w at peak	M_z
PHA	50 188	1.576	117 720	74 321
PHA–DMI	105 743	1.094	130 476	113 918
PHA–PEG	61 196	1.330	75 798	108 110
PHA–DMI–PEG	68 957	1.220	72 372	105 037
PHA–DMI–post-PEG	70 715	1.227	74 861	109 741

Fig. 4 PHA–PEG suspension in a water : acetone mixture (3 : 2). On the left the suspension formed by the PHA coupled with PEG; on the right the control vial with the PHA and PEG 4000 where the polyester was completely insoluble (glued to the magnet).

PHA–DMI–PEG. With the aim to obtain an amphiphilic PHA–PEG copolymer ready for click-functionalization, the enzymatic synthesis of a ter-copolymer PHA–PEG–DMI was investigated. Preliminary experiments were conducted to evaluate the best molar ratio between PEG4000 and DMI. Once the amount of PEG4000 was fixed (600 mg, 150 μmol), two parameters were considered: the saturation of hydroxyl ends, and the MW distributions of the resulting copolymers. 1 : 1, 1 : 2 and 1 : 4 PEG : DMI molar ratios were tested. Theoretically 100% saturation was calculated considering 4000 Da as the molecular weight of PEG and following a grade of conversion by the ^{1}H NMR peaks of ester protons centred at 4.28 and 4.34 ppm. The equimolar conditions lead to only a partial conversion of PEG4000, while the other two ratios were able to completely esterify the OH– end groups of PEG4000. Among the three tested conditions, a different distribution of *n*-meric species was observed; in particular, species with the highest MW were obtained for the condition with the lower amount of dimethyl ester (Table 5).

A 1 : 2 PEG : DMI ratio was selected for PHA coupling reactions as a good compromise between PEG4000 conversion and the number of monomers for the obtained products. The PHA–DMI–PEG reaction was firstly performed using a one-pot strategy. The GPC analysis revealed the formation of copolymers in which only one equivalent of the PHA was combined with PEG4000 and DMI (Table 5). It was presumably caused by the surplus of PEG4000 molecules which entropically hampered the DMI mediated PHA homo-coupling observed in the PHA–DMI reaction. A two-step reaction was therefore carried out to enhance the formation of high molecular weight species and prove the hypothesis. The PHA was firstly reacted with DMI (PHA–IA–PHA synthesis), and after 72 h, PEG and a supplementary enzyme were added without a purification step (PHA–DMI–post-PEG). Unexpectedly, no PHA homo-coupling in the final product was observed. It was probably the result of a nucleophilic attack of the OH– end group of PEG4000 to the PHA–DMI–PHA carbonyl bridge. So, the high molecular species formed during the first stage would be transesterified by the addition of PEG during the second step. The only difference between the two approaches for the tri-substrate reaction was observed in the ^{1}H NMR spectrum. In particular, the intensity of the peaks of the two possible CH_2 of PEGylate DMI, which is equal in the case of the PEG–DMI reaction, changes towards the relative intensity of 65 : 25 for the one-pot reaction and 75 : 25 for the two-step reaction. Although the data obtained from the partially purified product must be further investigated, the displacement to one signal represents a strong clue of an induced regio-specificity for the two-step approach (Fig. S8.†)

Table 5 Weight fraction of *n*-meric PEG : DMI species (n_x < n_y < n_z) from reactions with different reagent molar ratios. The M_n of detected species was 5261; 13 546 and 27 474 g mol^{-1} for n_x, n_y and n_z, respectively

	Weight fraction of *n*-meric PEG : DMI species		
PEG : DMI ratio	n_x	n_y	n_z
1 : 1	0.42	0.30	0.28
1 : 2	0.65	0.25	0.10
1 : 4	0.88	0.12	0.00

Experimental

Materials

All chemicals, solvents and enzymes were obtained from Sigma-Aldrich Chemical Company (St Louis, USA). Yeast extract and tryptone were purchased from Difco (Detroit, USA). Alfa Aesar sodium octanoate 96% was obtained from VWR (Radnor, USA).

Bacterial strains and growth conditions

E. coli Top 10 (Life Technologies, Monza, Italy) was used as a host for gene cloning. *E. coli* BL21 (DE3) (Novagen, Germany) and *E. coli* LS5218 (CGSC, Yale University) were chosen as hosts for recombinant biopolymer production. All bacterial strains were routinely grown in Luria–Bertani (LB) broth[47] at 37 °C, supplemented with ampicillin (100 μg mL^{-1}) if necessary. LB and M9 media were prepared and used as previously described.[47] M9 was supplemented with 0.01 mM of $FeSO_4$ (M9*) which is required by the enzymes of the tricarboxylic acid cycle and aerobic respiration chain.[48]

pPTSPHA construction

The oligonucleotide primers tac_promoter_Fw and tac_promoter_Rev were designed on the pFLAG-CTS™ sequence to drive the amplification of the tac promoter sequence and the upstream region from pFLAG-CTS™, herein defined pcr-tac. Amplification was performed using a High Fidelity Q5® polymerase (New England Biolabs, Ipswich, Massachusetts, USA) according to the manufacturer's instruc-

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tions. Sequences of suitable restriction sites were included in the primer design for cloning in pET16SPHA.[31] The oligonucleotides used were the following: tac_promoter_Fw (5′-ATGATTGCATGCGATCCGGGCTTATCGACTGCACGG-3′) and tac_promoter_Rev (5′-AATCATCCATGGATATGATATCTCCTGTG TGAAATTG-3′). The vector was hydrolyzed with *Sph*I and *Nco*I (Promega Italia, Milan, Italy) and ligated to the properly digested DNA fragment pcr-tac. Standard methods were employed for DNA manipulation.[47,49] The cloned PCR product was checked by sequencing (PRIMM Sequencing Service, Milan, Italy). Recombinant plasmids were transformed in *E. coli* strains using standard transformation methods.[47] The new two strains harboring the obtained pPTSPHA vector were named *LipoB* (*E. coli* BL21 (DE3)) and *LipoC* (*E. coli* LS5218). The *E. co*li LS5218 strain (*F*+, *fadR601*, *atoC512*(Const)) has an altered regulation of a fatty acid β-oxidation pathway which confers higher tolerance to fatty acids compared to BL21 (DE3).[50]

Table 6 Plasmids and strains

Plasmid or strains	Relevant characteristics/sequences	Source or reference
Plasmids		
pET16SPHA	pET16b derivative, *B. cereus* 6E/2 *sphaRBCBc*	31
pFLAG-CTS™	*ampr*, Ptac, pBR322 origin	Sigma-Aldrich
pPTSPHA	pET16SPHA derivative, alternative promoter sequence (*tac*)	This study
Strains		
E. coli Top10	F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Life Technologies
E. coli BL21 (DE3)	F⁻ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
E. coli LS5218	*F*′, *fadR601*, *atoC512*(Const)	50
LipoA	*E. coli* BL21(DE3), pET16SPHA (*sPha*)	31
LipoB	*E. coli* BL21(DE3), pPTSPHA	This study
LipoC	*E. coli* LS5218, pPTSPHA	This study

PHA production from recombinant *E. coli* strains

For PHA production, recombinant *E. coli* strains were cultured in 250 mL Erlenmeyer flasks containing 50 mL of sterile growth media. The flasks were incubated at 37 °C on a rotary shaker at 220 rpm. Sodium octanoate was prepared in a stock solution of 40 g L^{-1} and sterilized by filtering. The additional carbon source was added to the cultures in the reported concentrations (0.1–0.6% w/v) as extra-volume. The induction of protein expression was performed after 5 h from the inoculum with 0.5 mM IPTG (isopropyl-β-1-D-thiogalactopyranoside). The cultures were carried out for additional 48 h. Media composition: MM (per Liter): 1 g tryptone, 1 g NaCl, and 0.5 g yeast extract; M9Y1, M9Y2 and M9Y4 were prepared by the addition of yeast extract (YE), respectively, 1, 2, and 4 g L^{-1}, to M9* medium; MR_1 (pH 7): 13 g KH_2PO_4, 1.2 g $MgSO_4{\cdot}7H_2O$, 1.7 g citric acid, 4.0 g $(NH_4)_2SO_4$, 5.2 g K_2HPO_4, 20 g glucose, 2.0 g yeast extract, and 2.0 g tryptone; MR_2 (pH 7): 13.5 g KH_2PO_4, 4.0 g $(NH_4)_2SO_4$, 5.2 g K_2HPO_4, 0.8 g $MgSO_4{\cdot}7H_2O$, 1.9 g citric acid, and 20 g glucose. For all media, the pH of the saline solution containing phosphate salts and ammonium salts was adjusted before autoclaving. The media were completed after sterilization with a carbon source and other components were prepared in a concentrated solution. A stock solution (20 g L^{-1}) of yeast extract was autoclaved and then added to complete medium. Two-stage cultivation was performed with a protocol adapted from Taguchi *et al.*: recombinant strains were grown in 50 mL LB medium for 7 h at 37 °C, and IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.5 mM when the OD600 nm reached ~0.4. After the first step in rich medium, the cells were harvested and washed using distilled water, transferred to the same volume of MM containing sodium octanoate 2 g L^{-1}, and incubated at 37 °C for an additional 48 h (Table 6).[34]

Bioreactor fermentations were carried out in an Eppendorf NewBrunswick, BioFlo/CelliGen®115. The vessel was autoclaved using only water and the medium was complete after autoclave sterilization by adding a sterile concentrated stock solution of salts, carbon sources and yeast extract. An OD600 nm of 0.1 was set as a value for the inoculum; induction was performed after 5 h from the inoculum with 0.5 mM IPTG. The agitation rate was set to the value of 280 rpm and the air inject value was set to 1.3 dm^3 min^{-1}. The temperature was set to 37 °C. In all the cases, the effective induction of protein expression was verified by performing SDS-PAGE analyses according to the standard methods.[49]

Analysis of PHA production by the GC-MS method

PHA analysis on lyophilized cells was performed as previously described.[51] Calibration curves were obtained by using either 3-hydroxybutyrate (Sigma-Aldrich) or 3-hydroxyhexanoate (Larodan Fine Chemicals, Sweden) supplemented with benzoic acid as the internal standard. %PHA was expressed as the ratio between the produced PHAs (mg) and the CDW (mg) of the lyophilized cell material.

PHA extraction

Extraction was performed on lyophilized cells by using Soxhlet apparatus with chloroform. The dissolved biopolymer was concentrated and PHAs were precipitated by the dropwise addition of cold methanol (10 volumes). The polymer was collected by centrifugation (6000 rpm, 30 min and 4 °C), recovered using fresh chloroform and dried under a N_2 flux at 21 °C. The purified polymer was stored at 4 °C until further use.

PHA film preparation

PHA films were prepared as previously described by Pappalardo *et al.* with some modifications. Briefly, a solution of the polymer in toluene (50 mg mL^{-1}) was gently placed on the water surface (drop casting) in a Petri dish (ID 15 cm), and toluene was left to evaporate at 21 °C for 48 h.[36] The film was then collected on a glass slide and vacuum dried at 25 °C for 24 h.

^{1}H NMR

Nuclear magnetic resonance ^{1}H measurements were performed on a Bruker Avance II 400 spectrometer (resonance frequency 400.13 MHz for ^{1}H) equipped with a 5 mm observe broadband probe head (BBFO) with z-gradients. $CDCl_3$ was used as a solvent if not otherwise specified.

ATR FT-IT and FT-Raman analysis

ATR FT-IR spectra were recorded on a PerkinElmer Spectrum GX spectrometer. The ATR accessory (supplied by Specac Ltd, UK) contained a diamond crystal. A total of 6 scans for each sample were taken with a resolution of 4 cm^{-1}. All spectra were recorded at 21 °C and a wavelength interval between 4000 and 650 cm^{-1}. The FT-Raman spectra were recorded using a PerkinElmer Raman station 400, 166 coupled with a 785 nm laser. Spectra were recorded at a resolution of 2 cm^{-1} for 10 scans. All spectra were normalized in the region of 2200–2400 cm^{-1} before any data processing.

Hydrophobicity measurements

The hydrophobicity of the samples was quantified using water contact angle (WCA) measurements as previously described.[52] Polymer films were analyzed with the Drop Shape Analysis System DSA 100 (Kruss GmbH, Hamburg, Germany) using ddH_2O as a test liquid with a drop size of 5 μL. The WCA was measured after 1 s from the deposition of the drop and the data were analyzed using a video recording method. The data were obtained from the average of five measurements.

DSC and TGA

Differential scanning calorimetry was performed by using TA Universal Q2000 apparatus. 5–7 mg of the sample was heated from −40 to 200 °C at 10 °C min^{-1} under a nitrogen atmosphere. Thermogravimetric analysis (TGA) was performed on a Mettler TGA under a nitrogen atmosphere from 40 to 600 °C at 20 °C min^{-1}.

GPC

Gel permeation chromatography (GPC) was performed to determine the molecular weight distributions of the polymers, using a GPC Max Viscotek system equipped with a TDA 305 (refractive index, viscometer, low angle light scattering, and right angle light scattering), a UV detector and Phenomenex Phenogel columns: a precolumn and two columns, respectively, 10^6 and 10^3 g mol^{-1}. Samples were dissolved and eluted in chloroform stabilized with amylene or tetrahydrofuran stabilized with BHT. The method parameters were as follows: for chloroform triple point calibration using the polystyrene (PS) standard having a narrow molecular weight distribution (M_w 10 4959, M_w/M_n 1.037); and for tetrahydrofuran conventional calibration using PS standards having narrow molecular weight distributions (M_p: 3 279 807, 1 290 000, 560 000, 382 000, 102 543, 66 000, 22 000, 18 340, 11 600, 7500, 4900, 935). The analysis parameters were as follows: for chloroform 0.8 min ml^{-1}, 100 μL volume injection, 35 °C columns and detectors, and runtime 50 min; and for tetrahydrofuran 0.6 min mL^{-1}, 75 μL volume injection, 35 °C columns and detectors, and runtime 60 min. Sample preparation: after complete dissolution under mechanical stirring (about 5 mg mL^{-1}), they were filtered using 0.22 μm PTFE membranes.

Enzymatic functionalisation

Enzymatic functionalisation reactions were conducted similarly as previously reported by Corici *et al.*[44] with the following variations. Toluene was used as a solvent in order to ensure efficient mass transfer and completely dissolve the reactants. 200 mg of mcl-PHA and 600 mg pf PEG 4000 and different amounts of dimethyl itaconate (DMI) were used together with 10% w/w (on the total amount of reagents) of a commercial immobilized lipase B from *C. antarctica* (also known as Novozyme 435). Reactions were conducted in 50 mL round bottom flasks connected to a rotary evaporator at 50 °C. After 72 h, the reaction mixture was recovered with toluene and the products were characterized after the filtration of the biocatalyst using a cellulose filter. For PHA–PEG derivatives, a preliminary enrichment of the products aimed to remove the unreacted low molecular weight PEG was applied. The used method was similar to that reported by Ravenelle *et al.*:[23] the product mixture was first dissolved in hot dimethylformamide (DMF), and then the solution was cooled to 21 °C. Under vigorous agitation, double distilled water was slowly added until the final DMF concentration was approximately 10%.[23] The suspension was then transferred to a centrifugal filter device in regenerated cellulose with 10 000 MWCO (Amicon® Ultra-15 10K, Merck Millipore). After ultrafiltration, the product (suspension or precipitate or both) was freeze-dried and a white fluffy powder was recovered.

Conclusions

In this work, different recombinant *E. coli* systems were designed to allow the production of PHAs with high mcl moieties. Media optimization and system engineering were applied yielding up to 260 mg L^{-1} of PHB-*co*-PHHx which was characterized by a low grade of crystallinity and hydrophobic features (WCA of 90°). An enzymatic strategy for the functionalisation of PHAs with unsaturated moieties based on enzymatic coupling was shown, circumventing *in vivo* incompatibilities of the desired monomers. Indeed, polymer tunability was achieved by the enzymatic coupling of DMI, while the range of the applicability of a biocompatible telechelic polymer was widened by the coupling to hydrophilic PEG 4000. Bio-polymers with both functionalities (DMI and PEG) were obtained *via* a bio-catalysed reaction without any alteration in the MW of the starting PHA. Functionalization with an unsaturated methyl ester leads to the formation of a clickable long chain PHA while the reaction with PEG resulted in a suspension forming material in a hydrophilic environment. PEGylated PHB-*co*-PHHx copolymers have shown better performances for application in tissue regeneration and tissue compatibility

compared to PHB-*co*-PHHx and PHB,[11,53,54] and in the preparation of biomaterials for injectable drug delivery systems and wound healing.[46,55] Despite the promising results, further studies are indeed needed to modulate the MW distribution acting on the reaction parameters such as substrate concentration, temperature, time and biocatalysts.

Conflicts of interest

The authors declare no conflict of interests.

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2. Novel strategies for synthesis, functionalization and degradation of bio-based materials

His-Tag Immobilization of Cutinase 1 from Thermobifida cellulosilytica for Solvet-Free Synthesis of Polyesters

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His-Tag Immobilization of Cutinase 1 From *Thermobifida cellulosilytica* for Solvent-Free Synthesis of Polyesters

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For many years, lipase B from *Candida antarctica* (CaLB) was the primary biocatalyst used for enzymatic esterification and polycondensation reactions. More recently, the need for novel biocatalysts with different selectivity has arisen in the biotechnology and biocatalysis fields. The present work describes how the catalytic potential of *Thermobifida cellulosilytica* cutinase 1 (Thc_Cut1) was exploited for polyester synthesis. In a first step, Thc_Cut1 was immobilized on three different carriers, namely Opal, Coral, and Amber, using a novel non-toxic His-tag method based on chelated Fe(III) ions (>99% protein bounded). In a second step, the biocatalyzed synthesis of an array of aliphatic polyesters was conducted. A selectivity chain study in a solvent-free reaction environment showed how, in contrast to CaLB, Thc_Cut1 presents a certain preference for C_6–C_4 ester-diol combinations reaching monomer conversions up to 78% and M_w of 878 g mol^{-1} when the Amber immobilized Thc_Cut1 was used. The synthetic potential of this cutinase was also tested in organic solvents, showing a marked activity decrease in polar media like that observed for CaLB. Finally, recyclability studies were performed, which showed an excellent stability of the immobilized Thc_Cut1 (retained activity >94%) over 24 h reaction cycles when a solvent-free workup was used. Concerning a practical application of the biocatalyst's preparation, the production of oligomers with M_n values below 10 kDa is usually desired for the production of nanoparticles and for the synthesis of functional pre-polymers for coating applications that can be crosslinked in a second reaction step.

1. Introduction

The first reports on enzymatic synthesis of polyesters date back to the1990s, when respective groups led by Kobayashi, Seppälä, and Dordick reported the possibility of using biocatalysis in organic media for polymer synthesis. These pioneering works shed light on two enzymatic polymerization mechanisms: polycondensations and ring opening polymerizations (ROP). Regarding polycondensation reactions, halogenated diesters were the election choice for obtaining both aliphatic and aromatic polyesters using tetrahydrofuran,[1] diphenyl ether,[2] or pyridine[3] as solvent, while ROP reactions were carried out using lactones of various ring-sizes in bulk[4] or using combinations with polycondensation reactions.[5] For these initial reports, enzymes belonging to the protease and lipase families were mainly used in their lyophilized powder form.[1,3]

In the early 2000s, research interests shifted to enzymatic polymerization of polyesters toward the production of functional polymers such as aliphatic polyesters carrying lateral hydroxy[6] and epoxy[7] groups suitable for further functionalization or cross-linking. Moreover, reports by Sheldon et al.[8] inspired a tremendous increase in the investigation of enzyme immobilization techniques that would allow a simple recovery of the enzyme from the reaction media and its recyclability. Thanks to its promiscuity and activity in the most disparate reaction conditions, the lipase B from *Candida antarctica* (CaLB) emerged in these studies as the most used and investigated biocatalysts for polymerization reactions.[9]

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More recently, the interest in biocatalysis for polyester (and polyamide) synthesis[10,11] has again led to the investigation of fungal cutinases as compared with the more commonly known CaLB as biocatalysts for polycondensation and ROP reactions, especially exploiting renewable resources that would contribute to the closure of the polymers carbon cycle.[12,13] The first reports of cutinase-catalyzed synthesis of polyesters were published by the Gross group, which reported how cutinase from *Humicola insolens* (HiC) in its immobilized form was able to catalyze ROP of various lactones (both in bulk and in toluene),[14,15] as well as catalyze the polycondensation of aliphatic polyesters starting from AA-BB building blocks and ω-hydroxyacids.[16] Similarly, the cutinase 1 from *Thermobifida cellulosilytica* (Thc_Cut1) immobilized on epoxy-activated beads was reported for the synthesis of aliphatic polyesters in solvent-free reactions and organic media (toluene) starting from dimethyl adipate and various linear polyols (C_4–C_{12}).[17,18] In addition to these efforts for the synthesis of various polyesters, the group led by Katja Loos focused on the potential of cutinase for the synthesis of aliphatic[19] and aromatic[20] polyamides using cutinase from *Fusarium solani* pisi (Fsp) immobilized on Lewatit beads and in the form of cross-linked enzyme aggregates (CLEA).

The present work describes our investigation of the Thc_Cut1-catalyzed synthesis of biobased aliphatic polyesters starting from various dimethyl esters (C_4–C_8) and linear polyols (C_4 and C_8) in solvent-free systems. An innovative technology based on the coupling of the His-tag of the recombinant enzyme with the non-toxic Fe(III) chelated on the controlled porosity glass carriers developed by EnginZyme was used for the enzyme's immobilization, and the differences among three carriers with different superficial hydrophilicity (Opal-hydrophilic, Coral-hydrophobic, and Amber-semi-hydrophobic) were documented and exploited.

2. Experimental Section

Chemicals and Reagents: Opal, Coral, and Amber controlled pore glass beads were obtained from EnginZyme (Stockholm, Sweden) with the following specifications: Opal: hydrophilic surface, glass; Coral: hydrophobic surface, polymer; Amber: semi-hydrophilic, polymer; with pore diameters of 500 ± 50, 300 ± 50, and 300 ± 50 Å, respectively. The particle size was 75–125 μm for all three carriers. Dimethyl succinate (C_4, DMS), adipate (C_6, DMA), and suberate (C_8, DMB) were purchased from Sigma–Aldrich, and 1,4-butanediol (BDO), and 1,8-octanediol (ODO) were purchased from Merck. All other chemicals and solvents were also obtained from Sigma–Aldrich at reagent grade, and used without further purification if not otherwise specified.

Production, Purification, and Immobilization of Thc_Cut1 on Fe(III)-Activated Beads: The recombinant *T. cellulosilytica* cutinase 1 (Thc_Cut1) was produced in *Escherichia coli* and purified as previously described by Herrero Acero et al.[21] The Opal, Coral, and Amber beads were washed both with double distilled H_2O (2 times) and with the immobilization buffer (0.1 M Tris–HCl pH 7) (2 times), and then dried under vacuum for 48 h prior to use. A total of 1.0 g of dry beads were suspended in 10 mL of 1 mg mL^{-1} enzyme solution in 0.1 M Tris–HCl buffer pH 7 at 21 °C for 24 h on a blood rotator. Samples were withdrawn over time. The progress of the immobilization was monitored by evaluating the residual activity and protein concentration in the supernatant. After the immobilization, the enzyme preparations were extensively washed with 0.1 M Tris–HCl buffer pH 7 in order to remove all the non-ionic bound protein adsorbed on the support and then dried for 48 h under vacuum prior to use.

Enzymatic Activity and Protein Concentration Assays: Enzymatic activity (PNPB assay) and protein concentration determination according to the BioRad protein assay (Bio-Rad Laboratories GmbH, Vienna, Cat.No: 500-0006) were carried out as previously described by Pellis et al.[22]

Enzymatic Polycondensation Reactions: Enzymatic polycondensation reactions were achieved as previously reported by Pellis et al. using a thin-film reaction system.[23,24] Equimolar amounts (0.006 mol) of diester and diol were used together with a 10% w w^{-1} (on the total amount of monomers) of immobilized iThc_Cut1 preparations. Reactions were conducted in 50-mL round bottom flasks connected to a rotary evaporator, applying reduced pressure (535 mbar) as previously reported.[18] After 24 h, the reaction mixture was dissolved in THF (when the solvent workup was used) and the biocatalyst was filtered off using a cellulose filter. THF was then removed via rotary evaporation. The solvent-free workup instead consisted in a direct filtration of the still warm and therefore viscous liquid) reaction products (in order to remove the immobilized biocatalyst) using a cotton filter. For the recycling experiments the two above mentioned methods were used and the reaction products were characterized via ^{1}H-NMR and GPC without any further purification steps.

Gel Permeation Chromatography (GPC): Samples were dissolved in THF (250 ppm BHT as inhibitor). GPC was carried out at 40 °C on an Agilent Technologies HPLC System (Agilent Technologies 1260 Infinity) connected to a 17369 6.0 mm ID × 40 mm L HHR-H, 5 μm Guard column and a 18055 7.8 mm ID × 300 mm L GMHHR-N, 5 μm TSKgel liquid chromatography column (Tosoh Bioscience, Tessenderlo, Belgium) using THF (250 ppm BHT as inhibitor) as a mobile phase solvent (flow rate: 1 mL min^{-1}). An Agilent Technologies G1362A refractive index detector was employed for detection. The molecular weights of the obtained polymers were calculated using polystyrene calibration standards (250–70,000 g mol^{-1}).

Nuclear Magnetic Resonance: ^{1}H measurements were performed on a Bruker Avance II 400 spectrometer (resonance frequency 400.13 MHz for ^{1}H) equipped with a 5 mm observe broadband probe head (BBFO) with z-gradients. $CDCl_3$ was used as solvent unless otherwise specified.

3. Results and Discussion

3.1. His-Tag Based Immobilization of Thc_Cut1 on Various Carriers

After successful expression and purification, *T. cellulosilytica* cutinase 1 (Thc_Cut1) was immobilized on three different Fe(III)-carrying glass carriers with different superficial hydrophilicity: Opal-hydrophobic, Coral-hydrophobic, and Amber-semi-hydrophobic). The results presented in **Figure 1** show that the enzyme quickly coupled with Coral and Amber,

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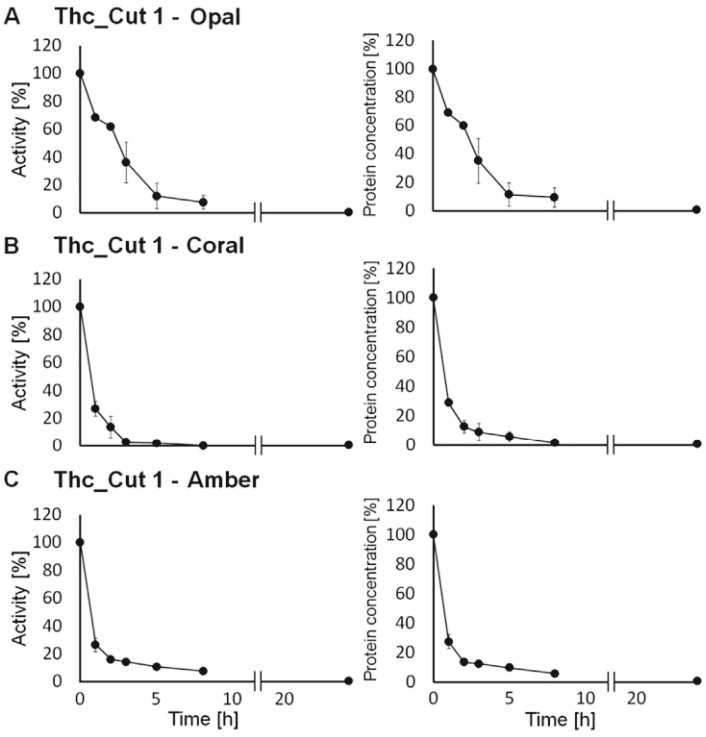

Figure 1. Immobilization profile of Thc_Cut1 on Opal A); Coral B); and Amber C) beads according to relative remaining cutinase activity (left) and protein concentration (right) in the supernatant. All reactions were performed in duplicates. Blank reactions showed no change in supernatant activity and concentration over time. The figure shows the mean ± SD.

leading to 70% binding within 1 h (Figure 1B and C), while after the same time period, only 30% of Thc_Cut1 had bound to the Opal carrier (Figure 1A). Interestingly, despite the significant difference in binding between Opal and Coral/Amber after 1 h of reaction, no differences were detectable among the three carriers after 24 h, with >99% of the biocatalyst successfully coupled to the beads. The exact values of the time-course monitoring of the Thc_Cut1 immobilization are reported in **Table 1**.

This different immobilization kinetic might be due to the fact that the Opal carrier is composed solely of glass beads (hydrophilic material), while the Coral and Amber are glass beads covered with a layer of polymeric material that make the beads hydrophobic and semi-hydrophobic, respectively.

3.2. Enzymatic Solvent-Free Synthesis of Biobased Poly(1,4-Butylene Adipate) Oligomers

After successful immobilization of the biocatalyst on the carriers, the synthesis of poly(1,4-butylene adipate) (PBA) as an example of biocatalyzed, biobased synthesis in a solvent-free environment was performed, and the efficacy of the three different preparations in performing this polycondensation reaction was assessed.

As shown in **Figure 2**, the best results in terms of monomers conversion were achieved using Thc_Cut1-Coral and Thc_Cut1-Amber that gave comparable conversions of 76 and 78%, respectively, while Thc_Cut1-Opal was less active, giving a conversion of only 57% after 24 h (Figure 2). Interestingly, and despite the different hydrophobicity properties discussed above, the more active preparations for the synthesis were those showing a faster immobilization kinetic during the first hour (Coral and Amber) (see the above section describing His-tag based immobilization of Thc_Cut1 on various carriers beads). GPC analysis of the samples fully confirmed the ^{1}H-NMR-calculated monomers conversions, with the higher M_n and M_w obtained using Thc_Cut1 immobilized on Coral and Amber (**Table 2**).

This efficiency difference among the three preparations is not surprising, as it is widely known that each biocatalyst has a preferred immobilization support/protocol that leads to the best recovered activity and stability under operational conditions.[25,26] An assigned ^{1}H-NMR spectra is shown as example in Supporting Information Figure S1.

3.3. Synthesis of Aliphatic Polyesters Using Dimethyl Esters and Polyols of Various Chain Lengths (C_4–C_8)

The polycondensation of dimethyl esters with a chain length from C_4 to C_8 with the linear diols 1,4-butanediol (BDO, C_4) and 1,8-octanediol (C_8, ODO) were carried out after the enzymatic synthesis of the biobased poly(1,4-butylene adipate).[12] Notably, the highest monomers conversions and molecular masses were obtained by combining the C_6 diester (dimethyl adipate) with the C_4 diol (BDO), while all the other combinations gave lower monomers conversions and shorter polymeric chains (**Figure 3**A). As previously reported by Hunsen et al., HiC also shows a certain

Table 1. Immobilization of Thc_Cut1 on Opal, Coral, and Amber beads according to relative remaining cutinase activity and protein concentration in the supernatant.

	Opal		Coral		Amber	
Time [h]	Activity [%]	Conc. [%]	Activity [%]	Conc. [%]	Activity [%]	Conc. [%]
0	100	100	100	100	100	100
1	68	69	27	29	26	28
2	62	60	14	13	16	14
3	36	35	2	9	14	12
5	12	11	2	6	11	10
8	8	10	0	1	8	6
24	0	1	0	1	0	1

All reactions were performed in duplicate.

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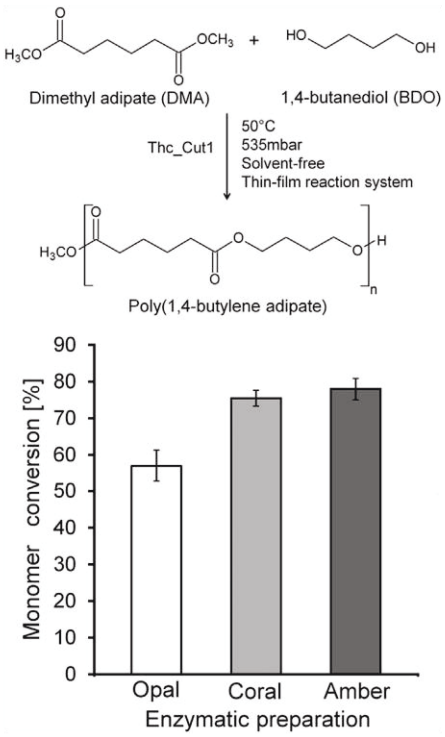

Figure 2. Synthesis of poly(1,4-butylene adipate) via Thc_Cut1 immobilized on three different supports: Opal-hydrophilic, Coral-hydrophobic, and Amber-semi-hydrophilic. All reactions were performed in duplicates. Blank reactions led to no detectable monomers conversion products. The figure shows the mean ± SD.

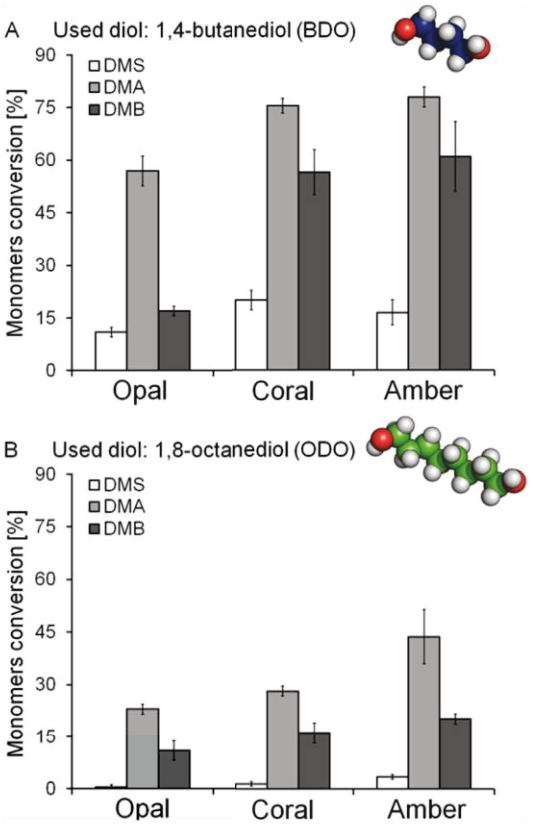

Figure 3. Synthesis of various aliphatic polyesters starting from succinic (DMS, C4), adipic (DMA, C6), and suberic (DMB, C8) dimethyl esters combined with 1,4-butanediol (BDO, C4) and 1,8-octanediol (ODO, C8) linear diols. A) 1,4-butanediol (BDO) and B) 1,8-octanediol (ODO). All reactions were performed in duplicates. Blank reactions did not lead to any detectable monomer conversion products. The figure shows the mean ± SD.

chain preference (C_{10}–C_{13} diacids for the polymerization with 1,8-octanediol), while CaLB shows greater substrate promiscuity.[16] From these results, we can conclude that, similar to HiC, the cutinase 1 from *Thermobifida cellulosilytica* has a defined chain selectivity for transesterification reactions.

Moreover, in the present study, we observed that the resulting monomer conversions were higher when the C_4 diol was used in combination with the C_4, C_6, and C_8 diester monomers than when combined with the C_8 diol (Figure 3B). These results are consistent with previous works reporting how the Thc_Cut1-catalyzed polycondensation of dimethyl adipate with C_6 and C_8 linear diols in a solvent-free system results in very low monomers conversions, while the C_4 diol shows higher conversion rates.[17]

The trend reported for the enzymatic polycondensation of PBA was further confirmed for all diesters-diols combinations, with Coral and Amber giving the best conversion rates in all cases, and Opal being the less active preparation for synthetic purposes (see Figure 3A and B).

Table 2. ^{1}H-NMR and GPC analysis of the poly(1,4butylene adipate) oligomers synthesized using Thc_Cut1 immobilized on three different supports.

Preparation	Conversion [%][a)]	M_w[b)]	M_n[b)]	$Đ$[b)]
Opal	57	633	506	1.25
Coral	76	786	581	1.35
Amber	78	878	597	1.47

Blank reactions let to no detectable polycondensation products.
[a)] Calculated via ^{1}H-NMR as previously reported[18,23]; [b)] calculated via GPC.

3.4. Biocatalysts preparations recyclability studies

The recyclability of the enzyme preparations was assessed using a solvent-based workup procedure and a solvent-free workup. When a 2Me-THF workup procedure was used, a marked conversion decrease was observed after the first 24-h reaction cycle, with a 42, 58, and 58% reduction for Opal, Coral, and Amber, respectively. After the second reaction cycle, the conversion rates were further reduced at 79, 86, and 64%, respectively (**Figure 4**). Similar to the simulations performed by

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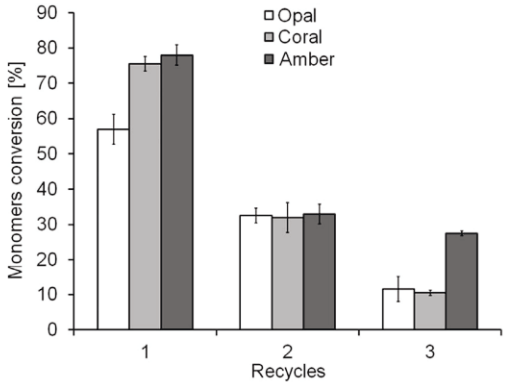

Figure 4. Evaluation of the recyclability of Thc_Cut1 immobilized on Opal, Amber, and Coral carriers in the synthesis of poly(1,4-butylene adipate) when a THF workup procedure was used. The recyclability was investigated for three reaction cycles, and is expressed as a percentage of BDO monomer reacted after 24 h of reaction. All reactions were performed in duplicates. Blank reactions led to no detectable monomers conversion. The figure shows the mean ± SD.

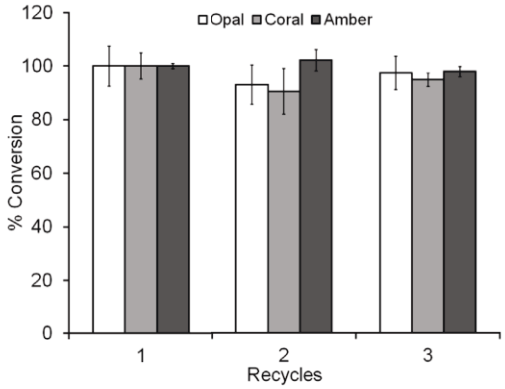

Figure 5. Evaluation of the recyclability of immobilized Thc_Cut1 in the synthesis of poly(1,4-butylene adipate) when a solvent-free workup procedure was used. The recyclability was investigated for three reaction cycles and is expressed as a percentage of BDO monomer reacted after 24 h of reaction. All reactions were performed in duplicates. Blank reactions led to no detectable monomers conversion. The figure shows the mean ± SD.

Li et al.[27] and those reported for CaLB,[28] these results indicate that Thc_Cut1 shows a lower stability in polar organic solvents, while being more active (or with a better preserved enzymatic activity) in apolar solvents such as toluene[17] and diphenyl ether.[10] The same has been observed for the members of the cutinase family Thc_Cut1, namely *Humicola insolens*[16] and *Fusarium solani* pisi.[20]

The analysis of the obtained molecular masses after each of the three reaction cycles is shown in **Table 3** and perfectly reflect what was observed with the ^{1}H-NMR monitoring of the monomers conversion rates, showing a progressive decrease of the obtained M_n and M_w for all three preparations, which is less accentuated for Amber in comparison with Opal and Coral.

The THF workup allows a simple removal of the biocatalyst formulation from the reaction products via a simple filtration step and solvent removal via rotary evaporator. The solvent can easily be recycled, as the ^{1}H-NMR analysis of the collected solvent after the reaction shows no contamination from monomers or oligomers. On the other hand, the enzyme loses the major part of its catalytic activity, therefore, showing no suitability for an industrial process in which the immobilized biocatalyst would be the most expensive component of the synthetic reaction system.[13]

In a second instance, a solvent-free workup was conducted. In this case, all three biocatalyst preparations demonstrated an excellent recyclability over the performed reaction cycles. An average retained activity of >94% was observed for all three preparations (**Figure 5**). Notably, the performed reusability study was carried out with 24 h-long reactions, while in past trials, the recyclability of Thc_Cut1 and CaLB immobilized on different carriers was investigated only on 3 h reactions.[17,23]

In addition to the recyclability studies, SEM characterization of the carrier's morphology was implemented in order to certify the suitability of the thin-film method for the polycondensation reactions. As with previous reports concerning rice-husk immobilized enzymes,[29] no detectable mechanical damages were observed to the carriers, nor a size reduction of the particles (Figures S2–S5). Concerning a practical application of the biocatalyst's preparation, the production of oligomers with M_n values below 10 kDa is usually desired for the production of nanoparticles[30] and for the synthesis of functional pre-polymers for coating applications that can be crosslinked in a second reaction step.[31]

Table 3. M_w and M_n analysis of PBA oligomers synthesized using immobilized Thc_Cut1 over three reactions cycles using a THF reaction workup.

	Preparation					
	Opal		Coral		Amber	
Cycle	M_w[a)]	M_n[a)]	M_w[a)]	M_n[a)]	M_w[a)]	M_n[a)]
1	633	506	786	581	878	597
2	371	348	463	408	436	425
3	306	282	330	297	410	326

Blank reactions led to no detectable polycondensation products.
[a)] Calculated via GPC.

4. Conclusions

In the present work, *T. cellulosilytica* cutinase 1 (Thc_Cut1) was successfully immobilized via His-tag on three different Fe(III)-activated carriers. While all three preparations turned out to be active for the synthesis of aliphatic polyesters in a solvent-free reaction system, the Amber and Coral-immobilized Thc_Cut1 showed the best results, yielding monomer conversions of around 80% after 24 h of reaction. Moreover, the recyclability of the preparation was investigated in both organic media (THF) and using a solventless approach, with the latter proving to be more suitable when an industrial scale-up is desired (retained

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activity of >94% for all three preparations). Further studies are ongoing on the immobilization of various biocatalysts on the Opal, Amber, and Coral carriers and their application for both polymerization reactions and conversion of small molecules carrying functional groups.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

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Authors Contributions

A. P., M. V., and F. Q. performed the experiments. A. P. planned the experiments. A. P. and G. M. G. wrote the manuscript.

Conflict of Interest

The authors declare no competing financial interest.

Keywords

enzymatic synthesis, cutinases, polyesters, green chemistry, enzyme immobilization

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2. Novel strategies for synthesis, functionalization and degradation of bio-based materials

Enzymatic Degradation of Aromatic and Aliphatic Polyesters by P. pastoris Expressed Cutinase 1 from Thermobifida cellulosilytica

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Enzymatic Degradation of Aromatic and Aliphatic Polyesters by *P. pastoris* Expressed Cutinase 1 from *Thermobifida cellulosilytica*

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To study hydrolysis of aromatic and aliphatic polyesters cutinase 1 from *Thermobifida cellulosilytica* (Thc_Cut1) was expressed in *P. pastoris*. No significant differences between the expression of native Thc_Cut1 and of two glycosylation site knock out mutants (Thc_Cut1_koAsn and Thc_Cut1_koST) concerning the total extracellular protein concentration and volumetric activity were observed. Hydrolysis of poly(ethylene terephthalate) (PET) was shown for all three enzymes based on quantification of released products by HPLC and similar concentrations of released terephthalic acid (TPA) and mono(2-hydroxyethyl) terephthalate (MHET) were detected for all enzymes. Both tested aliphatic polyesters poly(butylene succinate) (PBS) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) were hydrolyzed by Thc_Cut1 and Thc_Cut1_koST, although PBS was hydrolyzed to significantly higher extent than PHBV. These findings were also confirmed *via* quartz crystal microbalance (QCM) analysis; for PHBV only a small mass change was observed while the mass of PBS thin films decreased by 93% upon enzymatic hydrolysis with Thc_Cut1. Although both enzymes led to similar concentrations of released products upon hydrolysis of PET and PHBV, Thc_Cut1_koST was found to be significantly more active on PBS than the native Thc_Cut1. Hydrolysis of PBS films by Thc_Cut1 and Thc_Cut1_koST was followed by weight loss and scanning electron microscopy (SEM). Within 96 h of hydrolysis up to 92 and 41% of weight loss were detected with Thc_Cut1_koST and Thc_Cut1, respectively. Furthermore, SEM characterization of PBS films clearly showed that enzyme tretment resulted in morphological changes of the film surface.

Keywords: cutinase, *Thermobifida cellulosilytica*, *Pichia pastoris*, poly(3-hydroxybutyrate-co-3-hydroxyvalerate), poly(butylene succinate), poly(ethylene terephthalate), enzymatic hydrolysis, aliphatic polyesters

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INTRODUCTION

Plastic materials are ubiquitous in our daily life and although the annual European production is in a steady state since a decade, the global production is constantly increasing[1]. Most conventional plastics such as polyethylene, polypropylene, polystyrene, poly(vinyl chloride), and poly(ethylene terephthalate) (PET) are fully petrol-based and not biodegradable. The release of those plastic materials into the environment and their subsequent accumulation poses environmental risks and negatively impacts ecosystems, including the extreme consequences of plastic patch formation in rivers and oceans (Eriksen et al., 2014; Lechner et al., 2014). Considerable effort has been directed toward implementing bio-based plastics as environmentally-friendly alternatives to the traditionally petrol-derived materials. In particular, the substitution of polyesters such as PET and polybutyrate adipate terephthalate (PBAT) seems to be imminent since several market-leading companies are focusing their investigations on production of monomers derived from renewable biomass. Recent innovations also allow the biotechnological production of bio-based monomers from renewable carbon, enabling the replacement of petrochemical building blocks (Pellis et al., 2016c,d). These bio-based building blocks can be either produced by microbial conversions of various feedstocks or with combined biotechnological-chemical pathways that lead to various monomers such as 1,4-butanediol and adipic acid (used for the production of PBAT) (Harmsen et al., 2014). Fermentation of sugars or various other feedstocks, including lignocellulose (Pinazo et al., 2015), can also be used to obtain succinic and lactic acid for the production of poly(butylene succinate) (PBS) and poly(lactic acid) respectively. Poly(hydroxyalkanoates) (PHAs) on the other hand are directly produced by natural or engineered microorganisms. Mulch films are the most common and highly consumed plastic products in agriculture and their widespread use has led to an increase in environmental wastes. Therefore, commercially available mulch films are usually made of biodegradable plastics, with PBS as the main component (Koitabashi et al., 2012). In recent years there has been conservable interest in the substitution of PET with plant-derived poly(ethylene furanoate) (PEF) (Pellis et al., 2016b). The monomers for PEF (2,5-furandicarboxylic acid and ethylene glycol) can be 100% produced from renewable feedstocks (Pellis et al., 2016d).

The potential of enzymes for degradation of polymer building blocks has been studied by several groups. Various enzymes belonging to the cutinase family were reported to hydrolyze PET, the most used polyester (Yoon et al., 2002; Vertommen et al., 2005; Herzog et al., 2006; Heumann et al., 2006; Donelli et al., 2009; Herrero Acero et al., 2011; Ribitsch et al., 2011, 2012; Kanelli et al., 2015). Moreover, reports on the biocatalyzed hydrolysis of poly(lactic acid) (Pellis et al., 2015, in press; Ortner et al., 2017), poly(butylene succinate) (Hu et al., 2016) and poly(ethylene furanoate) (Pellis et al., 2016b) using similar biocatalysts were also described and certify the importance of such processes in the optics of a sustainable development (Clark et al., 2016; Pellis et al., 2016c). Despite the high industrial potential reported for cutinases from *Thermobifida* spp., these enzymes have usually been obtained by intracellular recombinant expression in *E. coli* (Herrero Acero et al., 2011; Su et al., 2013; Roth et al., 2014; Then et al., 2016), an approach that hampers the scale-up of the production process. Lately the methylotrophic yeast *P. pastoris* gained increasing interest as expression system for recombinant proteins for basic research as well as for industrial applications as shown by the number of filed patents (Bollok et al., 2009). In addition to the ability of *P. pastoris* to perform post-translational modifications one of the main advantages is that the recombinant proteins can often be secreted at high concentrations while maintaining their correct folding and activity (Cregg et al., 1993; Cereghino and Cregg, 2000; Ahmad et al., 2014; Hu et al., 2016). Furthermore, this host usually allows a simple production scale-up by changing from shaking flaks expressions to (fed-batch) fermenters (Schilling et al., 2002; Johnson et al., 2003; Zhao et al., 2008). Several commercial proteins are produced in *P. pastoris*, including recombinant *Tritirachium album* Proteinase K (Thermo Scientific, Waltham, MA, USA), Trypsin (Roche Applied Science, Germany), and nitrate reductase (The Nitrate Elimination Co., Lake Linden, MI, USA; Ahmad et al., 2014). In the past, successful expressions of cutinases from *Fusarium solani* (Kwon et al., 2009; Hu et al., 2016), *Alternaria brassicicola* (Koschorreck et al., 2010), *Glomerella cingulata* (Seman et al., 2014), and *Trichoderma harzianum* (Rubio et al., 2008) in *P. pastoris* have been reported. In this study, cutinase 1 from *Thermobifida cellulosilytica* (Thc_Cut1) as well as two glycosylation knock out mutants (Thc_Cut1_ko) were cloned and overexpressed in *P. pastoris* and screened for their ability to hydrolyze the aromatic polyester (PET) and the aliphatic polyesters [Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and PBS].

MATERIALS AND METHODS

Chemicals and Reagents

Restriction enzymes, antarctic phosphatase, T4 DNA ligase as well as Endo H_f were obtained from New England Biolabs (USA). Synthetic genes of *P. pastoris* codon optimized Thc_Cut1 and glycosylation site knock out mutants (Thc_Cut1_ko_Asn and Thc_Cut1_ko_ST) cloned into pMK-T were ordered from GeneArt (Germany). Pro-Q® Emerald 300 Glycoprotein Gel and Blot Stain Kit (P21857), CandyCane glycoprotein molecular weight standard (C21852) as well as *P. pastoris* KM71H strain and expression vector pPICZαB were acquired from ThermoFisher Scientific (USA). *E. coli* XL-10 cells were purchased from Agilent (USA). PureYield™ Plasmid Midiprep System, SV Gel and PCR Clean-Up System Kits and Mini-PROTEAN® TGX (Stain-Free™) Precast Gels were obtained from Promega (Germany) or BioRad (USA), respectively. Peptone, Yeast extract and DifCo yeast nitrogen base were purchased from Becton Dickinson (USA) and Zeocin™ was obtained from Eubio (Austria). All other chemicals were of the highest available purity and ordered

[1]Plastics Europe Plastics—the Facts 2015, 2015 (Accessed May 17, 2016).

from Sigma-Aldrich. PET powder obtained from still water bottle from Cristaline® was kindly provided by Carbios (St-Beauzire, France) and was previously characterized (Gamerith et al., in press). PHBV was purchased from Metabolix while PBS was purchased from Sigma-Aldrich. The PBS material used for quartz crystal microbalance (QCM) experiments was obtained from BASF and the physicochemical properties of this polyester were previously reported (Zumstein et al., 2016).

Designing of thc_Cut1 Glycosylation Site Knock out Mutants

Using NetNGlyc 1.0 server (Technical University of Denmark) five possible N-glycosylation sites were predicted in the native Thc_Cut1 sequence (GenBank accession no. ADV92526.1). Asparagine (Asn) at amino acid position 10 is directly followed by a proline which makes glycosylation unlikely due to conformational constraints. Also for Asn at position 233 the glycosylation potential was lower compared to the other potential glycosylation sites according to the prediction. Therefore, the three glycosylation sites at Asn 29, Asn 49, and Asn 161 were knocked out by changing the nucleotide sequence accordingly, resulting in two triple knockout mutants (for details see **Table 1**). Synthetic genes of the designed glycosylation site knock out mutants (Thc_Cut1_ko) cloned into pMK-T were ordered from GeneArt.

General Recombinant DNA Techniques

All general recombinant DNA techniques described in this work were performed following previously reported standard protocols (Sambrook et al., 1989). Digestion of cloning vector (pMK-T) and expression vector (pPICZαB) were performed with *Not*I Hf and *Xho*I, dephosphorylation was performed by antarctic phosphatase and T4 DNA ligase was used for ligation according to manufacturer's protocols (New England Biolabs). Plasmids and DNA fragments were purified by PureYield™ Plasmid Midiprep System Kit or Wizard® SV Gel and PCR Clean-Up System Kit. After transformation of *E. coli* XL-10 cells and plasmid purification pPICZαB_Thc_Cut1 and pPICZαB_Thc_Cut1_ko constructs were sequenced by LGC Genomics in order to confirm the DNA sequence.

TABLE 1 | Detailed design of Thc_Cut1 glycosylation site triple knock out mutants (Thc_Cut1_ko).

Name	AA mutations			Nucleotide mutations	
	Position	From	To	From	To
Thc_Cut1_ko_Asn	29	Asn	Asp	AAC	GAC
	49	Asn	Asp	AAC	GAC
	161	Asn	Asp	AAC	GAC
Thc_Cut1_ko_ST	31	Ser	Ala	TCT	GCA
	51	Thr	Ala	ACT	GCA
	163	Ser	Ala	TCC	GCA

Transformation into *P. pastoris* KM71H and Screening of Transformants

Eighty Milliliter 80 μL of electrocompetent *P. pastoris* KM71H cells were transformed with *Sac*I-Hf linearized pPICZαB_Thc_Cut1 or pPICZαB_Thc_Cut1_ko by electroporation (MikroPulser™, Bio-Rad) according to the manual instructions. Transformed cells were spread on yeast extract peptone dextrose sorbitol medium agar plates [YPDS, 1% (*w/v*) yeast extract, 2% (*w/v*) peptone, 2% (*w/v*) glucose, 1 M sorbitol, 2% (*w/v*) agar] containing 0.1 mg/mL Zeocin™ and incubated at 28°C for 3–5 days. Transformants were cultivated in YPD medium in 96-deep-well-plates and screened for multi-copy integrants on YPD agar plates [1% (*w/v*) yeast extract, 2% (*w/v*) peptone, 2% (*w/v*) glucose, 2% (*w/v*) agar] containing 0.1–2 mg/mL Zeocin™. Stock cultures of selected clones were stored at −80°C.

P. pastoris Shaking Flask Fermentation

For enzyme production, 1 L baffled shaking flasks containing 250 mL of buffered glycerol complex medium [BMGY; 1% (*w/v*) yeast extract, 2% (*w/v*) peptone, 1% (v/v) glycerol, 3.4% (*w/v*) yeast nitrogen base, 4×10^{-5}% biotin, 100 mM potassium phosphate buffer pH 6.0] were inoculated with *P. pastoris* KM71H transformants and incubated at 28°C and 150 rpm for approximately 16–18 h. Cells were harvested by centrifugation (3,000 × g, 8 min, 22°C) and the cell pellet was re-suspended in one-tenth of the original volume (75 mL culture volume in 300 mL shaking flasks). Enzyme expression was induced by the addition of methanol to a final concentration of 1% (*v/v*). Methanol was added twice daily to a final concentration of 1% (*v/v*) to sustain the induction. During fermentation, samples were collected by centrifugation (14,000 rpm, 5 min, 22°C) and supernatants were stored at −20°C until further use. After up to 120 h of enzyme expression, cells were harvested by centrifugation (4,500 rpm, 4°C, 20 min) and the supernatant was stored at −20°C until protein purification.

Immobilized Metal Ion Affinity Chromatography for Enzyme Purification

The enzyme purification from the fermentation supernatants was performed via affinity chromatography (ÄKTA purifier, GE Healthcare) using HisTrap™ excel 5 mL columns (GE Healthcare). After sample loading (flow rate 2 mL/min) the column was washed with 7 column volumes (CV) of equilibration buffer (20 mM NaH_2PO_4, 500 mM NaCl, pH 7.4) followed by 3 CV of 1% elution buffer (20 mM NaH_2PO_4, 500 mM NaCl, 500 mM imidazole, pH 7.4). The enzyme was eluted with 45% elution buffer for 6 CV. Finally the column was washed with 100% elution buffer for 3 CV and stored in a 20% ethanol solution. Proteins were detected at 280 nm. SDS-PAGE analysis of purification fractions was performed in order to confirm the presence of Thc_Cut1 or Thc_Cut1_ko in the pooled fractions. PD-10 columns (Sephadex™ G-25 Medium, GE Healthcare) were used to exchange the buffer to 100 mM KH_2PO_4/K_2HPO_4 pH 7.0 before storage of purified proteins at −20°C until further usage.

Expression Analysis and Enzyme Characterization

SDS-PAGE and Glycostain Analysis

SDS-PAGE of fermentation supernatant samples withdrawn at different time points was performed according to standard conditions (Laemmli, 1970). After staining with Coomassie Brilliant Blue R-250, SDS PAGE gels were imaged using a ChemiDoc (Chemidoc™ MP Imaging System, Bio-Rad). Stain free SDS-PAGE gels were directly visualized using a ChemiDoc (Chemidoc™ MP Imaging System, Bio-Rad) without further treatment. Deglycosylation of Thc_Cut1 and Thc_Cut1_ko mutants was performed using Endo H_f according to manufacturer's instructions (New England Biolabs). Glycostain gels were prepared according to Pro-Q® Emerald 300 Glycoprotein Gel and Blot Stain Kit manual and detected by G-Box or hand-held UV lamp at 300 nm.

Protein Analysis

Total protein concentrations in the fermentation supernatants (from different time points) as well as protein concentrations of purified enzymes were determined using the Bradford assay (BioRad) according to manual instructions and using bovine serum albumin (BSA) as standard.

Esterase Activity Assay

Esterase activity of fermentation supernatants (from different time points) and of purified enzymes was measured using *p*-nitrophenyl butyrate (pNPB) as soluble substrate according to Gamerith et al. using the experimentally determined extinction coefficient ($\varepsilon = 9.7\,mL\ \mu mol^{-1}cm^{-1}$) (Gamerith et al., in press).

Enzymatic Hydrolysis of Polyester Substrates

For hydrolysis reactions, 50 mg of PET powder or 5 mg of aliphatic polyester powders (PHBV, PBS) were weighed and incubated with 5 μM of enzyme (Thc_Cut1 or Thc_Cut1_koST) diluted in a final volume of 1 mL in KH_2PO_4/K_2HPO_4 (1 M, pH 8.0). In case of PBS films, pieces of 0.5 × 1.0 cm were cut and washed in three serial steps (5 g/L Triton X-100, 100 mM Na_2CO_3, and ddH_2O; each for 30 min at 50°C and 100 rpm) prior to hydrolysis reactions in order to remove possible surface contaminations (Pellis et al., 2015, in press; Gamerith et al., 2016). Incubations were performed in 2 mL tubes at 100 rpm and 65°C for different time frames. The released acids, alcohols and oligomers, namely: terephthalic acid (TPA) and mono(2-hydroxyethyl) terephthalate (MHET) for PET; succinic acid (SA) and 1,4-butanediol (BDO) for PBS and 3-hydroxybutyric acid (3-HBA) for PHBV were analyzed by HPLC using either a diode array detector (DAD) or a refractive index detector (RI). As a blank, polyester substrates were incubated in KH_2PO_4/K_2HPO_4 (1 M, pH 8.0) without enzyme. Enzyme blanks were also performed by incubating 5 μM solutions of enzymes in KH_2PO_4/K_2HPO_4 (1 M, pH 8.0) without polyester substrate. All hydrolysis experiments were performed in triplicates.

Analysis of Soluble Monomers and Oligomers Released by High Performance Liquid Chromatography (HPLC-DAD or HPLC-RI)

HPLC-DAD Detection of TPA and MHET

HPLC analysis of released products upon enzymatic hydrolysis of PET was performed as recently described by Gamerith et al. (in press). Briefly, after enzyme treatment of polyester powders the enzyme was precipitated with ice-cold methanol. After acidification to pH 3.5, samples were centrifuged (Hettich MIKRO 200 R, Tuttlingen, Germany) at 14,000 rpm at 0°C for 15 min, filtered (0.45 μm nylon) and transferred to HPLC vials. For HPLC (Agilent Technologies, 1260 Infinity) analysis, a reversed phase column C18 (YMC 30, 250 × 4.6 mm ID, S-5 μm) was used. Analysis was carried out with constant 10% 0.01 N formic acid and starting with 85% water and 5% methanol, gradual (1 min) to 10% methanol, gradual (to 8 min) to 50% methanol and gradual (to 10 min) to 90% methanol, back to starting position with a 7 min post run. The flow rate was set to 0.85 mL min^{-1} and the column was maintained at a temperature of 40°C. The injection volume was set to 10 μL. Detection of the analytes was performed with a photodiode array detector (Agilent Technologies, 1290 Infinity II) at a wavelength of 241 nm. Standards of TPA and bis(hydroxyethyl)terephthalate (BHET) were prepared in KH_2PO_4/K_2HPO_4 (1 M, pH 8.0) in a range of 0.005–0.5 mM and treated the same way as samples.

HPLC-RI Detection of SA, BDO, and 3-HBA

HPLC-RI detection of released products from PHBV and PBS was performed as previously reported by Pellis et al. (2015). Briefly, hydrolysis samples were precipitated following the Carrez method and filtered through 0.45 μm Nylon filters (GVS, Indianapolis, USA). The analytes were separated by HPLC using refractive index detection (1100 series, Agilent Technologies, Palo Alto, CA) equipped with an ICSep-ION-300 column (Transgenomic Organic, San Jose, CA) of 300 mm by 7.8 mm and 7 μm particle diameter. Column temperature was maintained at 45°C. Samples (40 μL) were injected and separated by isocratic elution for 40 min at 0.325 mL min^{-1} in 0.005 M H_2SO_4 as the mobile phase. Standards of SA, BDO and 3-HBA were prepared in KH_2PO_4/K_2HPO_4 (1 M, pH 8.0) in a range of 0.5–100 mM and treated the same way as samples.

Enzymatic Hydrolysis Measurements using a Quartz Crystal Microbalance (QCM)

The hydrolysis of spin-coated PHBV and PBS thin films by Thc_Cut1 was measured by QCM as previously reported (Zumstein et al., 2016). In brief, we spin coated thin films from chloroform solutions containing the respective polyester (concentration: 0.5% w/w) onto the surfaces of gold-coated QCM sensors. After air-drying the sensors, they were incubated in a buffered solution [3 mM tris(hydroxymethyl)-aminomethane, 10 mM potassium chloride, pH 7.0] for 14 h. The sensors were subsequently mounted into the flow cells of a QCM instrument (model E4, Q-sense) and rinsed with buffered solution of the same composition at a volumetric flow rate of 20 μL/min

and a temperature of 40°C. Upon attaining stable resonance frequencies of the fundamental tone and several oscillation overtones, we switched to delivering solutions that contained Thc_Cut1 (2.07 μg/mL) but otherwise were identical in pH and ionic composition to the solutions used for equilibration. We subsequently monitored changes in the resonance frequencies over the course of the hydrolysis experiment and related these frequency changes to adlayer mass changes using the Sauerbrey equation. We used the fifth overtone of the oscillation for calculations and data plotting. To measure the fraction of coated polyester dry mass that was removed over the course of the hydrolysis experiment, we measured the resonance frequency of each sensor in air after the experiment as well as before and after the initial polyester spin coating step. We note that Thc_Cut1 used in QCM experiments was expressed in *E. coli*.

Scanning Electron Microscopy (SEM)

PBS films morphology was qualitatively assessed through scanning electron microscopy (SEM). Control PBS (without any enzymatic treatment) and enzymatically hydrolyzed films (after 24, 48, 72, and 96 h) were surface characterized. All SEM images were acquired collecting secondary electrons on a Hitachi 3030TM (Metrohm INULA GmbH, Austria) working at EDX acceleration voltage.

RESULTS AND DISCUSSION

Glycosylation Site Knock out Mutant design, Vector Construction, and Transformation in *P. pastoris*

In its natural hosts or when heterologously expressed in *E. coli* (Herrero Acero et al., 2011; Su et al., 2013; Roth et al., 2014; Then et al., 2016) *Thermobifida* spp. cutinases are not glycosylated. In contrast, expression in *P. pastoris* may lead to glycosylation which can have positive effects such as increased stability, as previously shown for a *Thermobifida* xylanase (Zhao et al., 2015), human aquaporin 10 (Öberg et al., 2011) and *Rhizopus chinensis* lipase (Yang et al., 2015). Shirke et al. also recently reported that glycosylation stabilizes a *P. pastoris*-expressed cutinase from *Thiellavia terrestris* by inhibiting its thermal aggregation (Shirke et al., 2017). On the other hand, glycosylation may also have negative effects, as shown for example for a lipase from *Rhizomucor miehei* which had decreased activity upon N-glycosylation (Liu et al., 2014). Therefore, it was important to investigate the influence of glycosylation on the activity and stability of Thc_Cut1 when expressed in *P. pastoris*. Hence, two glycosylation site triple knock out mutants were designed (**Figure 1**). The recombinant pPICZαB_Thc_Cut1 and pPICZαB_Thc_Cut1_ko plasmids contained the codon optimized gene of wild type or mutated Thc_Cut1, the methanol inducible alcoholoxidase 1 promoter (AOX1), the *S. cerevisiae* α-factor secretion signal, a C-terminal 6x His-Tag and a transcription termination signal. The tightly regulated AOX1 promoter holds advantages for overexpression of proteins since cells are not stressed by the accumulation of recombinant protein during growth phase. Even the production of proteins that are toxic to *P. pastoris* is possible by uncoupling the growth from the production phase (Ahmad et al., 2014). The most commonly employed method of generating multi-copy expression strains in *P. pastoris* is based on direct screening of transformants on agar plates containing increasing concentrations of antibiotics (e.g., 0.1–2 mg/mL of Zeocin™) (Ahmad et al., 2014). After successful transformation by electroporation the selection of Thc_Cut1 and Thc_Cut1_ko transformants yielded clones that might contain multi-copy integrations as shown by growth on 2 mg/mL Zeocin™. A direct correlation between copy number and expression level has been shown especially for intracellular expression (Vassileva et al., 2001; Marx et al., 2009), but this direct correlation is not necessarily valid for secreted proteins (Marx et al., 2009).

Analysis of Cutinase Expression in Shaking-Flasks

After successful transformation and screening on high Zeocin™ YPD agar plates the best growing *P. pastoris* KM71H transformants of each enzyme were chosen for enzyme production in shaking flasks. Enzyme expression was induced by the addition of methanol and during fermentation several

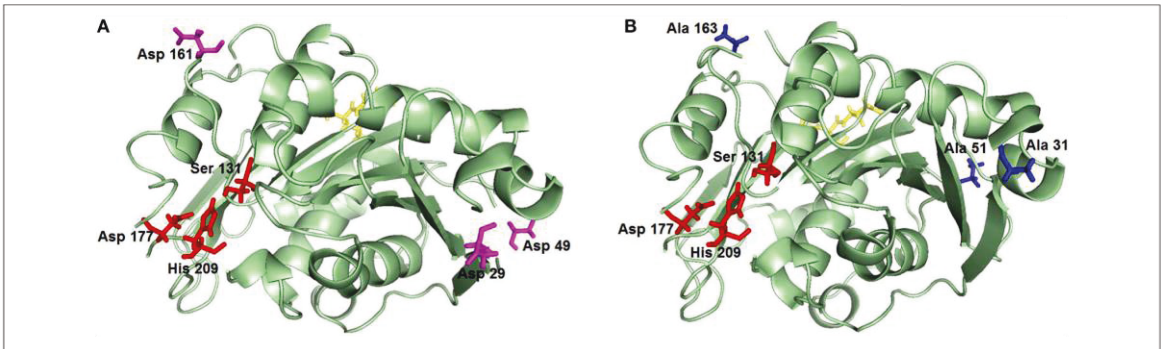

FIGURE 1 | Representation of Thc_Cut1 glycosylation site knockout mutants. (A) Thc_Cut1_koAsn, **(B)** Thc_Cut1_koST; mutated AA are in pink **(A)** and blue **(B)**, active site residues are in red, ALE linker sequence for 6×His-Tag is shown in yellow.

supernatant samples were collected by centrifugation. Analysis of these supernatant samples drawn at different time points during shaking flask fermentations by SDS-PAGE clearly showed that methanol induction successfully stimulated the expression of cutinases (**Figure 2**).

Although hyperglycosylation of heterologous proteins is more common in *S. cerevisiae* (Grinna and Tschopp, 1989), expression in *P. pastoris* can also lead to hyperglycosylation mainly attributed to N-mannosylation (Bretthauer and Castellino, 1999; Várnai et al., 2014). Glycosylation may affect the migration of the proteins on SDS-PAGE or, in case of heterogeneous glycosylation, may result in smears (Bretthauer and Castellino, 1999; Várnai et al., 2014). It was previously reported that heterologous expression of cutinase CUTAB1 from *Alternaria brassiciola* in *P. pastoris* led to a single band on SDS-PAGE when applied as crude supernatant. However, when applied after purification, an additional band became more distinct. Since purified and Endo H_f deglycosylated CUTAB1 only showed one band, the two different bands were assigned to the glycosylated and non-glycosylated enzyme (Koschorreck et al., 2010). In our case Thc_Cut1 appeared as a distinctive band around 38 kDa (**Figure 2A**, right), indicating its high level of glycosylation, whereas Endo H_f deglycosylated Thc_Cut1 showed one clear band corresponding to the calculated mass of 29.4 kDa (**Figure 2A**, left; Herrero Acero et al., 2011). The protein band around 70 kDa corresponds to Endo H_f used for deglycosylation. On the contrary, Thc_Cut1_ko mutants showed a clear band around 29 kDa with or without Endo H_f treatment suggesting that the glycosylation sites were successfully knocked out in both mutants (**Figures 2B,C**). These results were confirmed by staining SDS PAGE gels with Pro-Q® Emerald 300 glycoprotein stain, which creates a bright green-fluorescent signal on glycoproteins. For direct comparison of Commassie- and Glyco-staining, the same samples were loaded on two SDS PAGE gels whereas only one gel was glycostained afterwards. Glycostained gels showed clearly fluorescent bands of purified native Thc_Cut1 expressed by *P. pastoris*, whereas no fluorescent bands were detected for Thc_Cut1 expressed by *E. coli* (Gamerith et al., in press; **Figure 3**) or for Endo H_f deglycosylated Thc_Cut1 expressed by *P. pastoris* (see Figure S1).

Although all fermentation supernatant samples from different time points during expression of Thc_Cut1_ko mutants showed high unspecific fluorescent signals, no specific bands corresponding to Thc_Cut1 were detected, suggesting a high background noise of the medium (see Figure S2 for Thc_Cut1_ko_ST). Also purified glycosylation site knockout mutants did not show any fluorescent bands, verifying the successful knock out of all glycosylation sites (Figure S2, last lane for purified Thc_Cut1_ko_ST as example). Post-translational glycosylation processes might have an influence on the expression level due to their time- and energy-demand. Furthermore, also dissolved oxygen concentrations and careful control of the methanol levels are crucial for a high expression of recombinant proteins in *P. pastoris* (Seman et al., 2014). Methanol might not only have toxic effects for the cells but, as a highly flammable and hazardous substance it is also problematic for large-scale applications (Ahmad et al., 2014). Nevertheless,

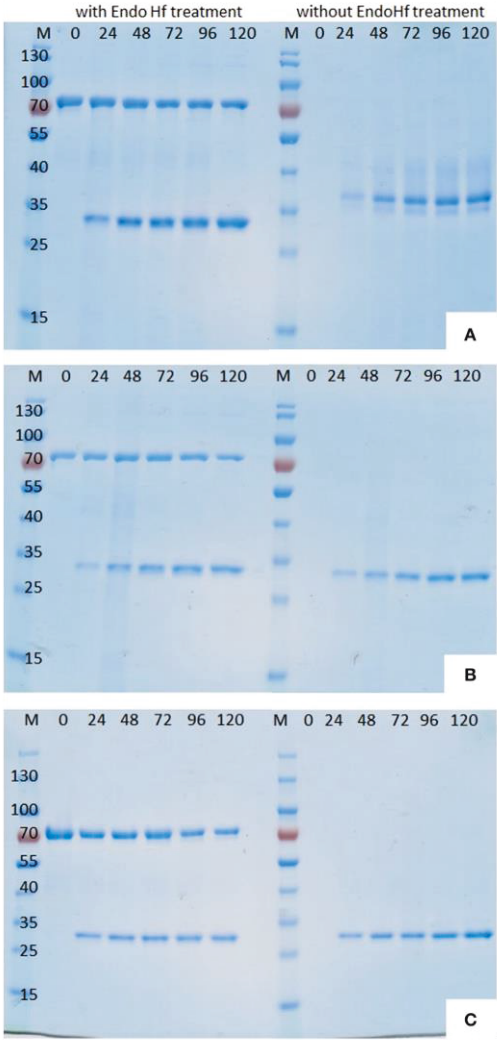

FIGURE 2 | SDS-PAGE (12%) of *P. pastoris* fermentation supernatant samples withdrawn at different time points after induction. (A) Thc_Cut1, **(B)** Thc_Cut1_koAsn, **(C)** Thc_Cut1_ko_ST; M = peqGold protein marker IV, #= time after methanol induction in h, each sample set was once loaded after treatment with Endo Hf (left) and once without Endo Hf treatment (right).

studies on methanol-inducible promoters, including AOX1, have shown that protein expression can also be achieved without methanol induction by constitutive co-expression of positively acting transcription factor Prm1p from either of the GAP, TEF, or PGK promoters (Takagi et al., 2012). In agreement with SDS-PAGE analysis also an increase of total extracellular protein concentration, determined by Bradford assay (BioRad) and (BSA) as standard, as well as an increase in volumetric esterase

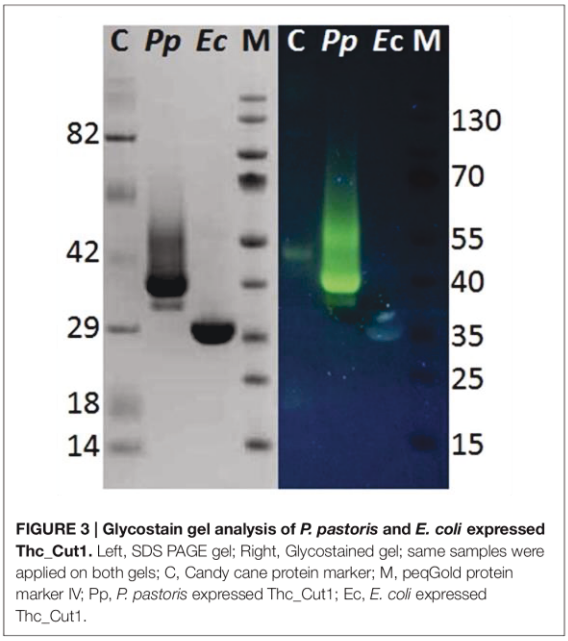

FIGURE 3 | Glycostain gel analysis of *P. pastoris* and *E. coli* expressed Thc_Cut1. Left, SDS PAGE gel; Right, Glycostained gel; same samples were applied on both gels; C, Candy cane protein marker; M, peqGold protein marker IV; Pp, *P. pastoris* expressed Thc_Cut1; Ec, *E. coli* expressed Thc_Cut1.

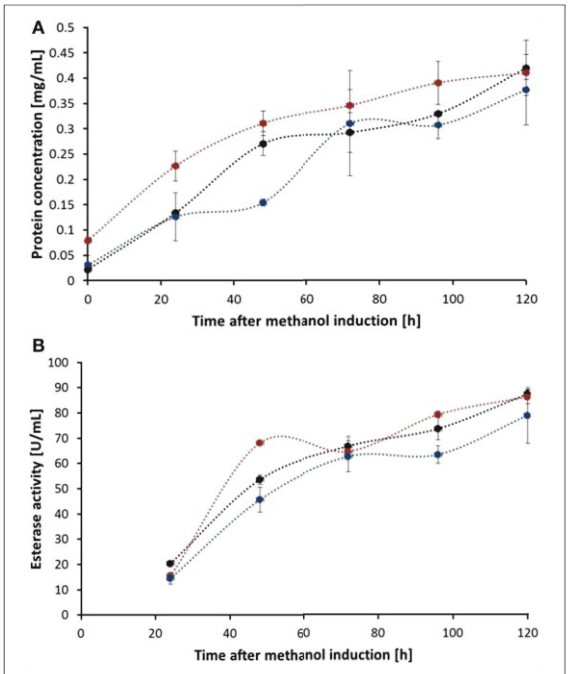

FIGURE 4 | Total protein concentration (A) and esterase activity (B) of fermentation supernatant samples of different time points after methanol induction. Thc_Cut1 (black line), Thc_Cut1_ko_Asn (blue line), and Thc_Cut1_ko_ST (red line). Time point 0 h indicates the start of methanol feed. Error bars show standard deviations of triplicate measurements.

activity on *p*NPB as substrate was detected in fermentation supernatants over time (**Figures 4A,B**, respectively). Within 24 h from the methanol addition, induction resulted in a clear increase in the total extracellular protein concentration. Interestingly, no significant differences between the expression of native Thc_Cut1 and Thc_Cut1_ko mutants concerning the total extracellular protein concentration were observed. Furthermore, the volumetric activity of all enzyme variants on *p*NPB was of the same order of magnitude.

Heterologous expression of Thc_Cut1 and Thc_Cut1_ko mutants in *P. pastoris* resulted in about 400 ± 20 mg total extracellular protein per liter in shaking flasks without optimization of culture conditions. These results are comparable to the previously reported expression level of *F. solani* cutinase in *P. pastoris* of 340 mg extracellular protein per liter (Kwon et al., 2009). In comparison to heterologous expression of CUTAB1 in *P. pastoris* by Koschorreck et al., yielding in 212 mg extracellular protein per liter, the expression level of Thc_Cut1 was almost doubled (Koschorreck et al., 2010). It is well-known that especially in the case of *P. pastoris* optimization of expression conditions in shake flasks or fed-batch fermenters can largely improve protein yields (Schilling et al., 2002; Zhao et al., 2008). However, the scope of this study was to demonstrate the general feasibility of Thc_Cut1 expression in *P. pastoris*.

Immobilized Metal Ion Affinity Chromatography (IMAC) for Enzyme Purification

From the 70 mL crude supernatants of each enzyme 65 mL were loaded onto HisTrap™ excel columns resulting in 14 mL of purified and buffer exchanged enzymes with different concentrations and esterase activities (**Table 2**). Interestingly, around 75% of the native Thc_Cut1 and Thc_Cut1_koST could be recovered from the crude supernatant, whereas only around 34% of Thc_Cut1_koAsn could be purified. Protein peaks of Thc_Cut1 and Thc_Cut1_ko were detected at 280 nm and the presence of enzymes in the corresponding fractions was confirmed by SDS-PAGE analysis (see Figure S3 for example of Thc_Cut1 purification).

Kwon et al. reported a negative effect of a C-terminal 6xHis tag on the cellular process for proper synthesis, folding, and secretion of *F. solani* cutinase in *P. pastoris* (Kwon et al., 2009). Similarly, also C-terminal fusion of small tags [such as FLAG-(Gly)5 and His-(Gly)5 tags] to the extracellular domain of human Fas ligand (hFasLECD) led to a failure in secretion of functional protein in *P. pastoris*, whereas the secretion of functional hFasLE CD was retained upon N-terminal tagging (Muraki, 2006). Nonetheless, since all cutinases used in this study had a C-terminal 6xHis tag, it was not possible to assess any effect of C-terminal tags within this study.

Enzymatic Hydrolysis of Aromatic Polyesters (PET)

Several cutinases (Vertommen et al., 2005; Heumann et al., 2006; Donelli et al., 2009; Kanelli et al., 2015)—including *E. coli*

TABLE 2 | Production of various ThC-Cut1 variants in *P. pastoris*.

Enzyme	Purification step	Total protein (mg)[a]	Total activity (U)[b]	Specific activity (U/mg)	Yield (%)[c]
Thc_Cut1	Crude supernatant	27	5,700	210	100
	Purified and buffer exchanged	21	2,100	100	75
Thc_Cut1_koAsn	Crude supernatant	25	5,100	210	100
	Purified and buffer exchanged	8	1,432	170	34
Thc_Cut1_koST	Crude supernatant	27	5,600	210	100
	Purified and buffer exchanged	20	3,500	180	75

[a]*Protein concentration was determined as described in section Protein Analysis and calculated for total volume.*
[b]*Esterase activity was determined as described in section Esterase Activity Assay and calculated for total volume.*
[c]*Relative yield with protein content of crude supernatant set to 100%.*

expressed Thc_Cut1 (Herrero Acero et al., 2011)—have been found to hydrolyze PET. For this reason, this aromatic polyester was chosen as substrate for performing the first hydrolysis experiments with cutinases expressed in *P. pastoris* in order to confirm their activity. Besides the crystallinity of polyesters (Mochizuki and Hirmai, 1997; Vertommen et al., 2005; Herzog et al., 2006; Mueller, 2006; Brueckner et al., 2008; Tokiwa et al., 2009; Pellis et al., 2016a), also the incubation temperature is well-known to affect the enzymatic hydrolysis of polyesters, mainly by affecting the polymer chain mobility (Marten et al., 2003; Eberl et al., 2009). Incubation temperatures close to the glass transition temperature (T_g) are suggested in order to promote enzymatic attack of polymers for degradation purposes (Mueller et al., 2005; Mueller, 2006; Kawai et al., 2014; Then et al., 2016) while $T < T_g$ are instead suggested when the surface hydrophilization is desired (Pellis et al., 2015, in press; Ortner et al., 2017). We recently reported that, for short term reactions, higher incubation temperatures led to faster hydrolysis rates of PET by *E. coli* expressed Thc_Cut1 (Gamerith et al., in press) while for longer reaction times limited enzyme stability may counteract this effect. Furthermore, the ionic strength as well as the buffer choice were found to have a severe effect on enzymatic hydrolysis of PET by polyester hydrolases (Schmidt et al., 2016). High buffer concentrations might prevent the pH decrease of the incubating buffer during hydrolysis reactions due to the acidic released products (e.g., TPA). Hence, hydrolysis of a 24% crystalline PET powder with *P. pastoris* expressed Thc_Cut1, Thc_Cut1_ko_Asn and Thc_Cut1_ko_ST were performed at 65°C in 1M KH_2PO_4/K_2HPO_4 pH 8.0 and released products were quantified by HPLC-DAD (**Figure 5**). No significant differences between the hydrolysis efficiency of the two glycosylation site knock out mutants could be observed, but treatment with the knock out mutants resulted in slightly increased TPA levels compared to the native Thc_Cut1. Up to 62 mM released TPA were observed after 96 h of hydrolysis, corresponding to ∼24% degradation of initial PET powder to soluble TPA. Compared to previously reported results for PET hydrolysis with Thc_Cut1 by Gamerith et al.—using incubation conditions of 100 mM KH_2PO_4/K_2HPO_4 pH 7.0 and 60°C (Gamerith et al., in press)—a combination of increased incubation temperature, higher pH and increased buffer concentration resulted in significantly higher hydrolysis rates of PET by Thc_Cut1 in this current study. This high degree of hydrolysis signifies a big step toward feasibility of enzymatic recycling of polyesters.

It is interesting to note that while the two glycosylation site knock out mutants showed very similar results with regards to both expression as well as PET hydrolysis rates, the purification yields of purified Thc_Cut1_ko_Asn were significantly lower compared to Thc_Cut1_ko_ST. Due to the highest productivity (and therefore scalability) for the expression, further enzyme selectivity studies on aliphatic polyesters were performed using the native Thc_Cut1 and Thc_Cut1_koST.

Enzymatic Hydrolysis of Aliphatic Polyesters (PHBV and PBS)

In order to investigate the substrate specificities of Thc_Cut1 and Thc_Cut1_koST in more detail, hydrolysis experiments were performed using the aliphatic polyesters PHBV and PBS as substrates. Both aliphatic polyesters were successfully hydrolyzed by Thc_Cut1 and Thc_Cut1_koST, although to very different extents. **Figures 6**, **7** show the quantified concentrations of released products after up to 96 h enzymatic hydrolysis of PHBV and PBS powders, respectively. Interestingly, Thc_Cut1 and Thc_Cut1_koST reached similar levels of released 3-HBA (∼0.5 mM) for the hydrolysis of PBHV, whereas for PBS, the released products were approximately twice as high for Thc_Cut1_koST as compared to native Thc_Cut1 (i.e., ∼14 vs. ∼7 mM SA and BDO). Due to different substrate concentrations (50 mg for PET vs. 5 mg for PBS and PHBV) the absolute values of released products seem lower compared to PET hydrolysis, but in fact the quantified SA and BDO concentrations correspond to ∼24 and 48% degradation of initial PBS powder to soluble released products by Thc_Cut1 and Thc_Cut1_koST, respectively. This finding indicates that there was a remarkable influence of the glycosylation on the substrate specificity. Glycosylation may not only lead to increased stability and protection against proteolysis, but may also have a role on the catalytic activity. For several proteases it has been reported that glycosylation can alter their substrate recognition, their specificity and binding affinity, as well as the turnover rates. Moreover, glycans which are in the vicinity of the active site are more likely to influence the substrate binding (Goettig, 2016). Recently, we have demonstrated that both surface engineering as well as attachment of polymer binding modules or hydrophobins can dramatically influence

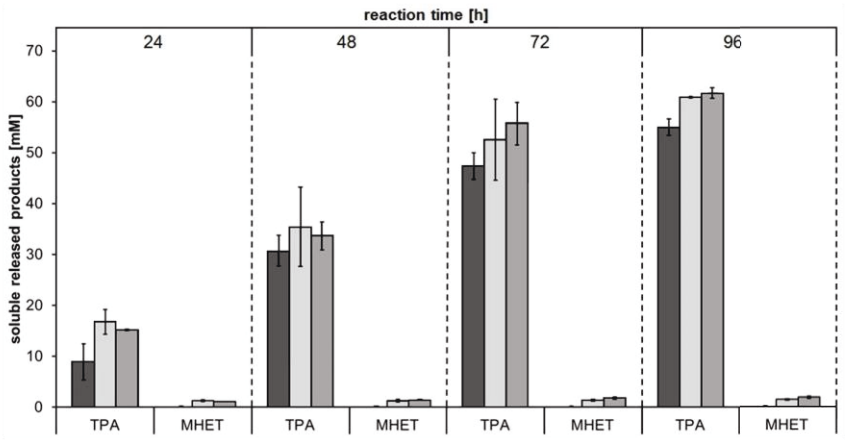

FIGURE 5 | Concentrations of soluble released products terephthalic acid (TPA) and mono(2-hydroxyethyl) terephthalate (MHET) upon enzymatic hydrolysis of PET powder by Thc_Cut1 (dark gray bars), Thc_Cut1_ko_Asn (light gray bars) and Thc_Cut1_ko_ST (middle gray bars). Time scan for 24, 48, 72, or 96 h was performed at 65°C with 5 μM enzyme in 1 M KPi pH 8.0 with 50 mg/mL substrate at 100 rpm.

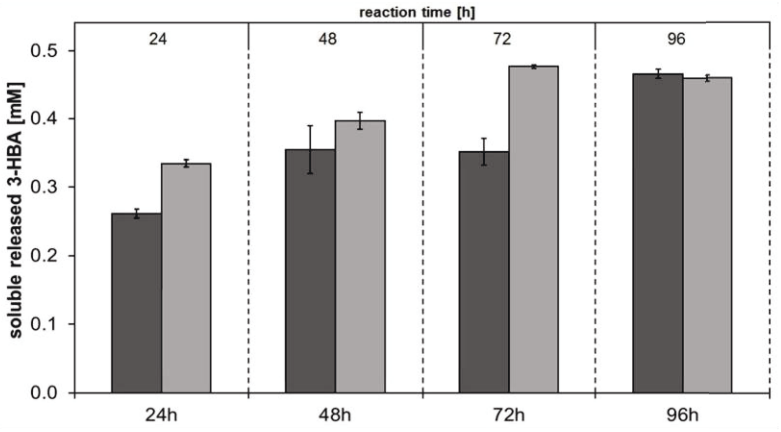

FIGURE 6 | Concentrations of soluble released 3-hydroxybutyric acid (3-HBA) upon enzymatic hydrolysis of PHBV powder by Thc_Cut1 (dark gray bars) and Thc_Cut1_ko_ST (middle gray bars). Time scan for 24, 48, 72, or 96 h was performed at 65°C with 5 μM enzyme in 1 M KPi pH 8.0 with 5 mg/mL substrate at 100 rpm.

sorption and thereby hydrolysis of polyesters (Herrero Acero et al., 2013; Ribitsch et al., 2013, 2015).

To support faster Thc_Cut1-mediated hydrolysis of PBS than PHBV, we complemented the data above by QCM measurements. Previous studies showed that QCM can be used to study both adsorption of enzymes to the polyester surface and mass loss of polyester films due to enzymatic hydrolysis in real time (Ribitsch et al., 2013; Perz et al., 2015; Zumstein et al., 2016). Here, we monitored the mass change of spin-coated PBS and PBHV films during their hydrolysis by Thc_Cut1 (**Figure 8**). These measurements showed that the mass of the spin-coated PBS films rapidly decreased after the addition of Thc_Cut1 and that the adlayer mass reached stable final values within 1.5 h of the onset of Thc_Cut1 addition (**Figure 8A**; results of duplicate experiments). When Thc_Cut1 was added to PHBV films, we measured an initial adlayer mass increase that we ascribed to the adsorption of Thc_Cut1 to the film surface. Detection of this mass increase implies that PHBV hydrolysis was slow (in contrast to PBS). Slow PHBV hydrolysis was substantiated by the finding of slow and continuous decreases in the PHBV film mass over the subsequent hours of continuous exposure to Thc_Cut1 (**Figure 8B**). The differences in the mass decreases determined

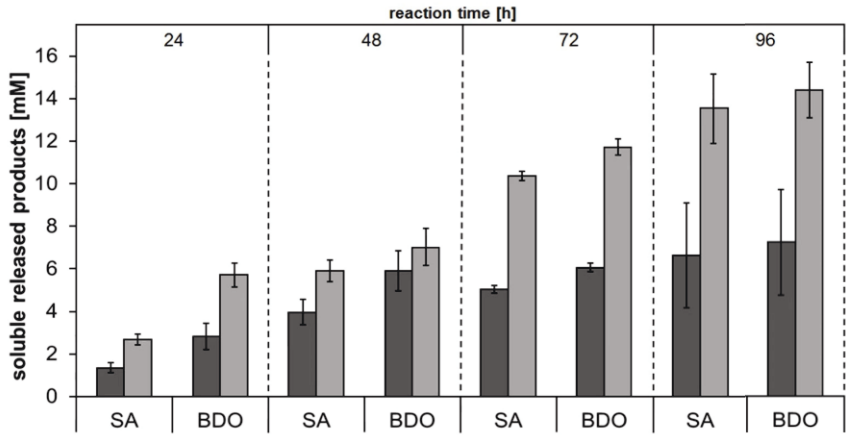

FIGURE 7 | Concentrations of soluble released products succinic acid (SA) and 1,4-butanediol (BDO) upon enzymatic hydrolysis of PBS powder by Thc_Cut1 (dark gray bars) and Thc_Cut1_ko_ST (middle gray bars). Time scan for 24, 48, 72 or 96 h was performed at 65°C with 5 μM enzyme in 1 M KPi pH 8.0 with 5 mg/mL substrate at 100 rpm.

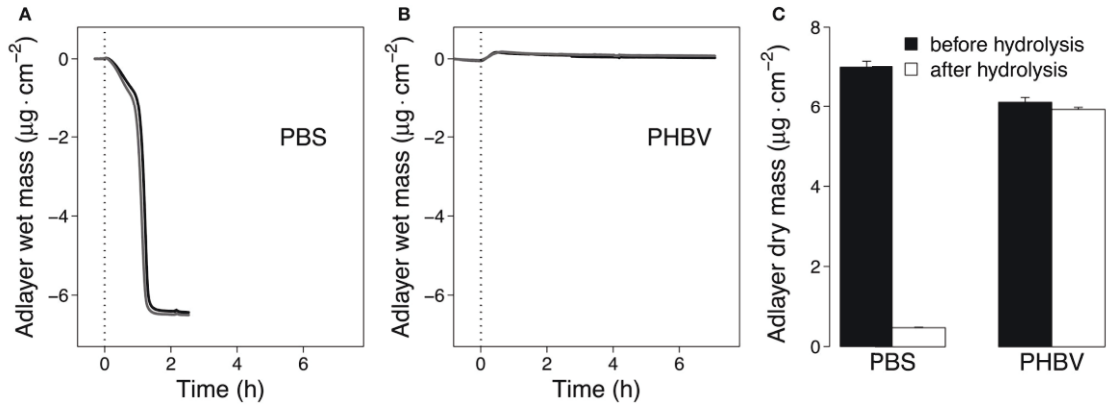

FIGURE 8 | Quartz Crystal Microbalance (QCM) measurements of hydrolysis of spin-coated PBS and PHBV films by Thc_cut1. (A,B). Progress curves of the polyester adlayer mass coated onto the QCM sensors during the hydrolysis of PBS **(A)** and PHBV **(B)**. At time $t = 0$ h we added Thc_cut1 to the buffer running over the films, as indicated by the vertical dashed lines in panels **(A,B)**. Black and gray lines represent duplicate experiments **(C)**. Dry masses of the sensor adlayers before and after the hydrolysis experiments. Hydrolysis experiments were performed at 40°C and pH 7.0 (3 mM Tris buffer).

in the QCM-D measurements were consistent with the changes in the dry masses of the sensors, which we determined by measuring the adlayer masses of dried sensors before and after spin coating step and after the enzymatic hydrolysis experiment. These measurements revealed that 93 and 3% of the spin-coated PBS and PHBV masses, respectively, were removed during the hydrolysis experiments. We note that the Thc_Cut1 that was used for the QCM experiments was expressed in *E. coli*. This Thc_Cut1 variant is expected to have no glycosylation and to therefore show the same activity on polyesters as the Thc_Cut1_ko_ST variant that was expressed in *P. pastoris*, for which we showed the absence of glycosylation (Figure S2). In summary, the QCM-based analysis supported faster hydrolysis of PBS than PBHV by Thc_Cut1.

Among the tested polyesters, PBS was most extensively hydrolyzed and was therefore chosen for additional analyses. Hydrolysis of PBS films followed by weight loss and SEM analysis were performed. The concentrations of released products from PBS films showed the same trend as for PBS powder—Thc_Cut1_koST released more than double the amount of hydrolysis products compared to native Thc_Cut1 (∼48–50 mM SA and BDO by Thc_Cut1_koST vs. ∼12–15 mM SA and BDO by Thc_Cut1) (**Figure 9** and Figure S4). These results are in accordance with the weight loss, which reached up to

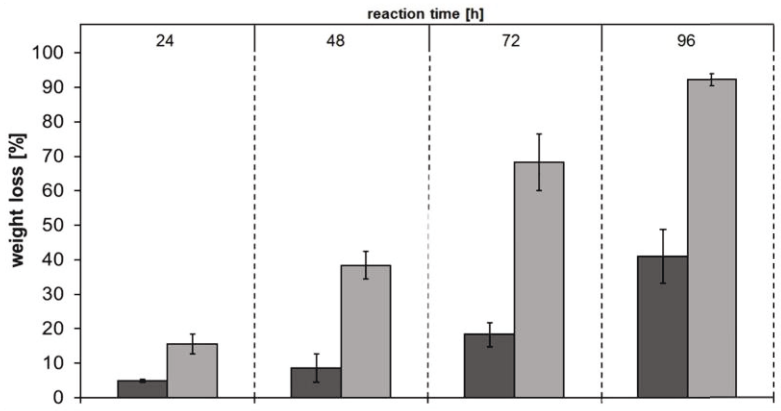

FIGURE 9 | Weight loss of PBS films upon enzymatic hydrolysis by Thc_Cut1 (dark gray bars) and Thc_Cut1_ko_ST (middle gray bars). Time scan for 24, 48, 72, or 96 h was performed at 65°C with 5 μM enzyme in 1 M KPi pH 8.0 with 0.5 × 1.0 cm PBS films at 100 rpm

92% with Thc_Cut1_koST and only around 41% with Thc_Cut1 within 96 h (see **Figure 9**). Hu et al. recently reported on complete degradation of PBS films by a recombinant cutinase from *Fusarium solani* within 6 h (Hu et al., 2016).

To complement the PBS hydrolysis data, an additional SEM characterization of the film surfaces was performed. **Figure 10** shows clear changes in the morphology of PBS film surfaces caused by treatments with both native Thc_Cut1 and Thc_Cut1_koST (**Figures 10C–F**), while no detectable changes of the control samples occured (**Figures 10A,B**). Moreover, 24 h of enzymatic hydrolysis of the PBS films surface resulted in more surface erosion when using the ko mutant than the native Thc_Cut1 (**Figure 10C** vs. **Figure 10E**). After 96 h, the formation of "holes" throughout the polymeric sample is visible for the ko mutant (**Figure 10F**) while only an increased surface roughness was observed for the Thc_Cut1 treatment (**Figure 10D**).

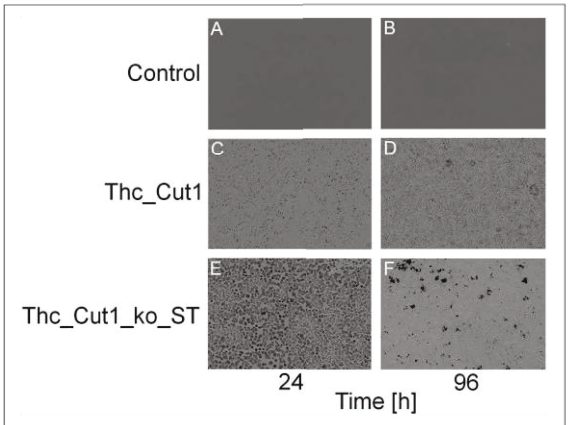

FIGURE 10 | SEM surface characterization of PBS films. (A,B) control reactions (polymer film + buffer only); **(C,D)** Thc_Cut1-catalyzed hydrolysis (polymer film + Thc_Cut1 in buffer); **(E,F)** Thc_Cut1_ko_ST-catalyzed hydrolysis (polymer film + Thc_Cut1_ko_ST in buffer). All samples were measured after 24 (left column) and 96 h (right column) of treatment. It's clearly visible the increase of the hydrolysis reaction from 24 to 96 h **(C–F)** and the higher damage to the film by Thc_Cut1_ko_ST that yield, after 96 h to holes that go throughout the film **(E)**.

CONCLUSIONS

In this study, we demonstrated the general feasibility of expressing Thc_Cut1 and two glycosylation site knock out mutants, Thc_Cut1_koAsn and Thc_Cut1_koST, in *P. pastoris*. Furthermore, we have shown that Thc_Cut1 and Thc_Cut1_ko mutants hydrolyze aromatic (PET) and aliphatic (PHBV and PBS) polyester powders, although at very different rates as shown by HPLC quantification of released products. These findings were also confirmed by QCM measurements, which showed a 3.0% mass change for PHBV thin films and a 93.2% mass decrease for PBS thin films upon enzymatic hydrolysis with Thc_Cut1. The finding that treatment of PBS films with Thc_Cut1 and Thc_Cut1_koST resulted in large PBS weight losses and clear effects on film surface topography imaged by SEM confirm the potential of Thc_Cut1 and mutants for degradation of PBS films. Together with the high activity of Thc_Cut1 and Thc_Cut1_ko mutants on PET, this study provides a significant contribution toward enzymatic degradation of polyesters.

AUTHOR CONTRIBUTIONS

CG, SG, and SZ expressed and purified the enzymes. DR designed the mutants. CG performed the PET hydrolysis experiments. MZ and MS conducted the QCM hydrolysis experiments and wrote the related sections of the manuscript. MV and AP performed

the aliphatic polyesters hydrolysis of powders and films and the relative SEM images. CG, AP, EH, and GG wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00938/full#supplementary-material

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3. Biorefinery for biomaterials: how to connect biodiesel industry and PHA production

WFOs in biodiesel industry and the developed Bioprocess

3.1 WFO in biodiesel industry and the developed Bioprocess

Fossil fuels, Greenhouse Gasses and Global Warming

The reduction of fossil fuel's consumption to reduce CO_2 emissions has become a crucial countermeasure for global warming. According to this, many international activities are underway in order to decrease greenhouse gases' emissions, such as the Kyoto Protocol of 1997 and the 2020 EU directive, which sets three specific targets that need to be achieved within 2020, such as producing at least 20% of UE energy from renewable sources, improving 20% the energy's efficiency and cutting off 20% of greenhouse gas emissions (from 1990 levels).

Biofuels as an alternative

Biofuels have been around as long as cars have. At the start of the 20th century, Henry Ford planned to fuel his Model T's with ethanol and early diesel engines were shown to run on peanut oil. However, discoveries of huge petroleum deposits kept gasoline and diesel cheap for decades and biofuels were largely forgotten until the recent fluctuation in oil prices, along with growing concern about global warming, caused by carbon dioxide emissions (Ottinger, 2009). Biofuels are fuels produced directly or indirectly from organic material (biomass), obtained through a process of biological carbon fixation. Burning a fuel made from plants has no overall effect on carbon dioxide in the atmosphere, because the CO_2 taken from the air as plants grow is then put back when the same biofuel is produced and burned. On the basis of biofuel production's source, they are classified as: i) first generation, ii) second generation, iii) third generation and iv) fourth generation. First Generation biofuels refer to the fuels that have been derived from sources like sugar, starch, vegetable oil/ animal fats, seeds and grains using conventional technologies. Wheat and sugar are the most widely used feedstocks in bioethanol production, while oil seed rape is the most used in case of biodiesel. First generation biofuels have a number of associated problems: they compete with global food market, demanding higher and higher volumes of crops, they represent an expansive option for energy security, when total production costs are taken into account, they don't always meet their claimed environmental benefits because the biomass feedstock may not always be produced sustainably and they compete for scarce water resources in some regions. To overcome these limits second generation biofuels have been developed: they are produced from non-food crops such as wood, organic waste, food crop waste and specific biomass crops, eliminating the main problem, food vs energy, related to first generation biofuels. Life cycle assessments of second-generation biofuels have also indicated an increase in 'net energy gains', preventing any unwanted increment in the CO_2 atmospheric level. Third generation biofuels are based on the use of engineered energy crops as energy source, such as algae, that can be cultured as low-cost, high-energy and entirely renewable feedstock. Algae, for example, offer the enormous advantage to grow on land and water unsuitable for food production, reducing the strain on already depleted water sources. Fourth Generation biofuels, instead, count on producing sustainable energy using genetic modifications to implement CO_2's capture and storage. Biomass materials, after absorbing CO_2 during their growth, are then converted into fuel through the same processes used for second generation biofuels. With this overview, it may seem that biofuel represents the ideal solution to all problems mentioned above but unfortunately the entire procedure that takes to produce it is not so "ideal". While biofuels may be cleaner to burn, there are strong indications that the process required to produce them - including the machinery necessary for crops' cultivation and plants' production - has

hefty carbon emissions. High costs of energy outputs, for refining biofuels and build the necessary manufacturing plants, have Also to be taken into account; and last but not least massive quantities of water are required for proper irrigation of biofuel crops, which could strain local and regional water resources. With more development and research, it could be possible to overcome biofuels' disadvantages, making them suitable for widespread consumer use. Only once these disadvantages will be minimized, biofuel's market will be able to show entirely its big potential. Much of this could rely on the ability of energy producers to discover better plants to raise for fuel that use less water, less land and grows quickly or on the use of waste materials, which can be used as cheap feedstock for biodiesel production. To be considered a valid alternative, any sort of biofuel besides having environmental benefits should also provide a net energy gain, be economically competitive and be producible in large quantities without reducing or competing with food supplies. In other words, the entire life-cycle of its production should be taken into account, when considering its use as a possible substitute. Based on these criteria, biodiesel has been identified as one of the most promising biofuels, able to yield 93% more energy than the one invested in its production (Popp et al., 2014).

Biodiesel

Biodiesel: is made from vegetable oils and animal fats, using a transesterification reaction aimed to production of fatty acid methyl esters (FAME). It has only slightly less energy than regular diesel and can be used as a fuel for vehicles in mixture with gasoline or in its pure form. However, since it's more corrosive to engine parts (gaskets and seals) than standard diesel, when blends with a biodiesel content higher than 30% are used, engines need to be properly designed. Usually it's used as a diesel additive to reduce levels of particulates, carbon monoxide and hydrocarbons from diesel-powered vehicles. Using 100-percent biodiesel (B100) eliminates all the sulfur emissions associated with conventional diesel, cuts emissions of carbon monoxide and smog-producing particulate matter almost in half and reduces hydrocarbon emissions by a range between 75 and 90%. Perhaps most significantly, using B100 reduces the emissions of carbon dioxide - the main greenhouse gas causing global warming - by more than 75%. Even using a blended biodiesel fuel like B20 (a 20% biodiesel/80% petrodiesel blend offered at most biodiesel fueling stations) still reduces carbon dioxide emissions by 10%. As a cleaner burning fuel, biodiesel is also better for a car's engine (with *ad hoc* gaskets and seals) than conventional diesel, providing greater lubrication and leaving fewer particulate deposits behind (Chattopadhyay and Sen, 2013).

Biodiesel production methods

Biodiesel can be produced through different kinds of methods such as preheating, blending, micro-emulsification, pyrolysis and transesterification (Hazar and Aydin, 2010; Nainwal et al., 2015; Bora et al., 2016; Abbaszaadeh et al., 2012; Bilgin et al., 2015). Among these methods, transesterification process was noted as the most suitable and simple method (Long and Fang, 2012). Transesterification is the process of displacement of the organic group of an ester with the organic group of an alcohol, in presence of a catalyst. In the transesterification of vegetable oils, a triglyceride reacts with an alcohol in presence of a strong acid or base, producing a mixture of fatty acids alkyl esters and glycerol.

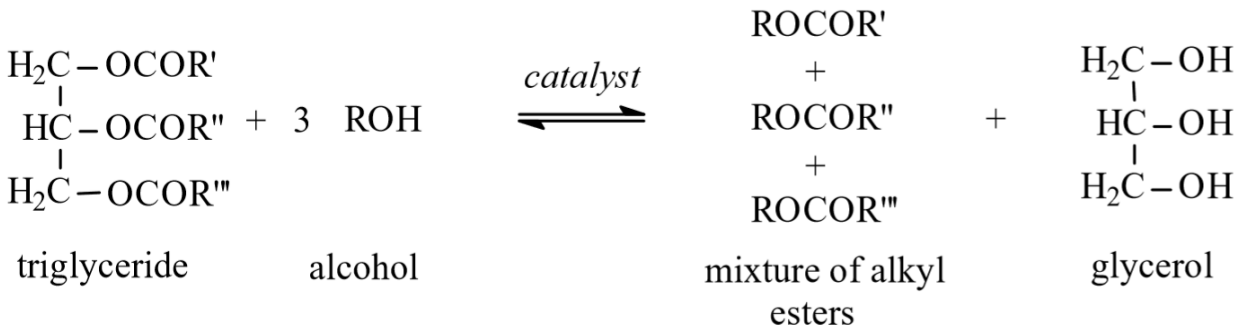


***Figure 3.1.1**: Scheme of transesterification reaction*

The overall process is a sequence of three consecutive reactions, in which di- and mono-glycerides are formed as intermediates. The stoichiometric reaction requires for each mol of a triglyceride 3 mol of the alcohol. However, since transesterification is a reversible reaction, alcohols are usually charged in excess to assist a rapid triglyceride conversion and ensure a high yield. Methanol and ethanol are the alcohols commonly used, especially methanol because of its low cost. The reaction's rate can be significantly improved by the presence of a catalyst. On the basis of the catalyst used, biodiesel's production methods can be distinguished in the ones that use: I) basic catalyst, such as sodium or potassium hydroxides, II) acid catalyst, such as sulfuric acid, III) enzymatic catalyst, such as lipases.

I. Base-catalyzed transesterification

The most commonly used base catalysts are potassium hydroxide (KOH) and sodium hydroxide (NaOH), along with an alcohol (methanol or ethanol), in a molar ratio that varies between 1:3 and 6:1. The amount of catalyst changes between 0.5 % to 1% w/w, while the reaction temperature ranges from 25 to 120°C, depending on the specific catalyst used. Hydrolysis of triglycerides and alkyl esters may occur if water is present, which leads to the formation of free fatty acids and thus to undesired soap. Saponification will also occur if a strong base reacts with esters and triglycerides directly. It has also been claimed that, when the FFA level exceeds 5 %, abundant soap's formation inhibits the separation between biodiesel and glycerol and decreases the yield of the final product (Canakci and Van Gerpen, 2001). In general, the use of a basic catalyst is more desirable since it provides the satisfactory conversion within a short time (Saifuddin et al., 2015).

II. Acid transesterification

Sulfonic (Stern, and Hillion, 1990) and sulfuric (Freedman et al., 1986) acids are the most common catalysts used in this procedure since they give very high yields in esters but it takes a lot of time for the reaction to reach complete conversion (almost more than 3h) (Freedman et al., 1984) and requires typically temperatures above 100°C. As in the alkaline reaction, an excess of alcohol ensures a better conversion in FAME. However, due to the fact that the reaction rate of a liquid acid catalyzed transesterification is slower than the one when an alkali-catalysts is used and due to their stronger corrosive nature, the use of liquid acids has never enjoyed the same popularity as its counterpart in the industrial process.

III. Enzymatic transesterification

Enzymatic production of biodiesel has many advantages over conventional methods as high yields can be obtained, ensuring lower reaction temperatures and easier ways to recover glycerol. For this purpose, microbial lipases are often used as powerful biocatalysts to generate an environmental friendly and economic fuel (Arthy

et al., 2014). However, limitations of this application are still high due to the high costs of the enzyme and high reaction time.

Transesterification related problems

Currently, most of the commercial biodiesel production is performed by the alkali-catalyzed transesterification, since it can be operated under mild conditions to achieve significant conversion with minimal side reactions and reaction time. Several aspects including the type of catalyst, alcohol/vegetable oil molar ratio, temperature, purity of the reactants (mainly water content) and free fatty acids (FFAs) content have an influence on the course of the transesterification. In particular, the last two parameters play a key-role in the success of the reaction having a negative influence when they exceed in the feedstocks (Oji and Emuraye, 2016). Water favors the formation of FFAs by hydrolysis of triglycerides and esters products (biodiesel). As a result, FFAs in presence of basic homogeneous catalysts generate soap, which creates serious problems in separating the product and ultimately hindering the catalytic activity (Oji and Emuraye, 2016). To minimize these inconveniences highly refined vegetable oils must be used for the process; otherwise, an additional step, conceived as a feedstock's pretreatment, could be taken into account when using untreated vegetable exhaust oils in order to reduce their acid and water concentrations under an optimum threshold limit, i.e., FFAs < 5 % and water < 0.5 %. Therefore, knowledge of different parameters of waste cooking oil needs to be accomplished in order to adjust them and making them suitable for the success of the transesterification reaction.

Reducing Biodiesel production's costs

The base cost of each gallon of biodiesel is the sum of raw materials costs (vegetable or animal oil feedstock, methanol, catalyst, heat), market influences of supply and demand and taxes. Additional items that affect the final cost are transportation, distribution, permits, state regulation and consistent quality assurance of the fuel. Among these, the cost of highly refined vegetable oils (used as feedstock) accounts for 80% of the total. Therefore, its substitution with vegetable exhaust oil, a more economical feedstock, could significantly lower its enormous prices of production. The term "vegetable exhaust oil (VEO)" refers to vegetable oil that has been used in food production and is no longer viable for its intended use. Since 2000, in Europe, a total of about 17 million tons vegetable oils are annually consumed and this amount raises approximately 2% each passing year (Agriculture and Food Development Authority, 2000). Waste oil arises from many different sources, including domestic, commercial and industrial ones and it represents a problematic waste stream since it requires to be properly managed. Its improper disposal in the drainage system might lead to its blockage (or even cause metal's corrosion and damaging issues) and huge investments need to be made in order to ensure proper cleanliness of the same. It may also prove to be disastrous if dumped onto the soil or if discarded into the nearest water body, since its eco-toxic proprieties could contaminate the soil and damage plants (Sanli et al., 2011). Therefore, the idea of the substitution mentioned above, which encourages biodiesel's production using waste vegetable oils, offers a triple-facet solution: economic, environmental and waste management (Wang et al., 2007).

Waste frying oils (WFO)

According to UK Environment Agency: "Waste cooking (frying) oils (WCO-WFO) are purified fats of plant or animal origin, which are liquid at room temperature. Like all fats, cooking oils are esters of glycerol and a varying blend of fatty acids, are biodegradable, insoluble in water, but soluble in organic solvent. Cooking oils are

generally processed and used in production of products fit for human consumption and do not contain toxic substances."

Quality of WFO

WFO quality is volatile not just because it is collected from different sources. The main parameters to determine the quality of WFO are the level of cleanliness, the level of Free Fatty Acids (FFA) and the water content. The cleanliness and the amount of FFAs depends on the products that are fried, the frequency of replacing WFO with fresh cooking oil and the vegetable oil used for cooking. Due to the collection from diverse gastronomic entities preparing different foods, the final WFO mix needs to be hydrated and filtered in order to produce biodiesel according to the EU standard EN14214. Purity of variety is only guaranteed by WFO from food processors or fast-food companies, which always use the same vegetable oil for the same products with strict internal regulations for replacement. On average 5-6% of pollutants such as small pieces of fried food need to be removed from the collected WFO.

Current WFO uses and their relative importance

Storically this waste raw material has been recycled in several processes including: i) animal feeding, ii) energy production, iii) oleochemistry and iv) biodiesel production. As a reaction to the BSE166 scandals in the early 2000s, since 31 October 2004 waste cooking oil from catering premises can no longer be used as an ingredient in animal feed. In the EU only WFO from food manufacturing, and fresh or unused cooking oil, can continue to be used in animal feed. This change has been introduced as a measure to safeguard animal health and the subsequent food chain under the Animal By-Products Regulation EC 1774/2002 (ABPR). WFO derived from other sources can be converted in ii-iv.

Regarding ii, the oleochemical industry relies on animal fats and WFO for the production of a variety of products ranging from consumer products like shampoo and candles, to plastics and building materials. According to APAG, the European association of the oleochemical industry, the relation between WFO and animal fats used in the industry is 1:9 (i.e. for every 10 tonnes of raw materials, 1 tonne is WFO and 9 tonnes is animal fat). The relatively low WFO share is explained by its variable quality, due to the variety of sources from different entities using different vegetable oils. For the oleochemical industry the carbon chain profile is important. Palm oil has a C14 chain, while rapeseed and soy have C16 and C18 chains respectively. A WFO mix could therefore comprise of carbon chains ranging from C14 to C18. Consistency of the raw material stream is only guaranteed by WFO that originates from food processors or fast-food companies, which always use the same vegetable oil inputs for the same products with strict internal regulations for replacement. Until 2003 it was difficult to process WFO in winter into standardised biodiesel, due to the high amount of palm oil in the WFO resulting in a higher Cold Filter Plugging Point (CFPP). Hence there was a shift of WFO from the biodiesel to the olechemical industry and to cogeneration units in winter. With the help of developed cold stabilizers this problem no longer exists and WFO with high palm content could be used for biodiesel production in winter (Toop et al., 2013). Furthermore ABPR 1069/2009 amended in April 2013, allows the use of animal fats of all categories for oleochemical products. This is of great importance as the oleochemical industry may rely on both animal fats and WFO, which could equally be substituted. In addition, the oleochemical industry prefers animal fats due to lower risk of contamination and constant quality. The more animal fat that is available for the oleochemical industry

the less WFO is needed, thereby increasing the share for the biodiesel industry. Ecofys estimates that 90% of the WFO in the EU-27 is currently used for biodiesel production and 10% is used by the oleochemical industry (Spöttle et al., 2013). The use of WFO for biodiesel offers a genuinely sustainable outlet for a problematic waste product and one with the potential to offer significant GHG savings. Further support to WFO conversion in biodiesel comes from Article 21(2) of the EU Renewable Energy Directive which allows Member States to count bio-fuels produced from wastes, residues, non-food cellulosic materials and ligno-cellulosic materials twice towards their 10% renewable energy in transport target for 2020 (http://data.europa.eu/eli/dir/2009/28/oj).

Low ILUC potential for WFO in the EU

According to the Low Indirect Impact Biofuel (LIIB) methodology, a waste or residue is low Indirect Land Use Change (ILUC) if no current uses of the material are displaced, other than current bio-energy uses or forms of disposal such as land-filling and burning. This means that the low ILUC potential of WFO is quantified by taking the total sustainable potential of WFO and deducting all current uses except bio-energy and dumping. In the EU-27 the alternative uses of WFO are biodiesel production or use for oleochemical products. According to LIIB incineration of WFO is not an alternative use and therefore does not have to be taken into account. As stated above the amount of WFO processed by the oleochemical industry is quite low. 90% of the estimated maximum WFO potential from gastronomy is used for biodiesel production already and its continued use for biodiesel, does therefore not impact other current WFO uses and can thus be classified as low ILUC risk. In addition the WFO imports can be added, due to their assumed use for biodiesel production. Altogether around 1 million tonnes of WFO could be processed into biodiesel in the EU-27 with a low ILUC risk. If the untapped potential of household WFO is taken into account, the potential for ILUC-free WFO use in the EU might total 3 million tonnes (Spöttle et al., 2013).

Raw material prices

The WFO market has changed dramatically over the past few years, although it is still relatively immature and can be intransparent. To restaurants or food processors the WFO is primarily a waste. Prices paid for WFO are therefore reported to vary widely. In 2008 the Environment Agency stated “some collectors charge to collect WFO, some collect for free and some pay the premises to receive their WFO”.

Price ($/L)	Source
0.20-1.20	https://www.alibaba.com/product-detail/Used-Cooking-Oil-Waste-Vegetable-Oil_50034804898.html?spm=a2700.7724838.2017115.81.X6lU37
0.35-0.40	https://www.alibaba.com/product-detail/Used-Cooking-Oil-Used-Cooking-Oil_50030046647.html?spm=a2700.7724838.2017115.301.JX9WtE
0.50-1.00	https://www.alibaba.com/product-detail/ISO-ISCC-Certification-Used-Cooking-Oil_50033948688.html?spm=a2700.7724838.2017115.89.X6lU37

***Table 3.1.1**: Overview of WFOs offers available at www.alibaba.com on July 2017.*

Competition in the WFO market is reported to have increased dramatically so much so many of the large WFO generating entities are already covered. In addition, an

increasing number of WFO thefts are reported in the EU, and also in the USA (Toop et al., 2013).

WFO supply chain

The WFO supply chain has not been highly regulated. It is a waste product and one that historically would typically be tipped into the drainage system by those who produce only small amounts. Restaurants are the major source for WFO followed by food processors and households. Within a specific sector such as restaurants there may also be a wide variation in the quantity and quality of the WFO produced, depending on the type of restaurant or the time of year. In the EU-27 the gastronomy sector is well covered by WFO collectors, with the exception of restaurants in remote rural areas. Recovery rates are expected to increase in areas not yet covered by WFO collection, especially for example in Eastern Europe, as long as economic incentives like double-counting justify the logistical effort. For the EU-27 Ecofys estimates a maximum collectable WFO potential in the gastronomy sector of 972,000 tonnes (Spöttle et al., 2013). Although restaurants are the primary source of WFO the potential significantly increases if food processors and households are also taken into account. Domestic sector was, and remains, a significant part of the remaining collection potential. It is not easy to access due to the large number of small point sources, but increasing numbers of local councils are beginning to offer collection points and several examples of domestic collection services are emerging, although it is still at a very small scale (only in the Netherlands, Spain and partly in Austria). Sometimes WFO collectors address WFO directly for biodiesel production, but often there are several stages involved in the collection and aggregation of WFO. There may simply be larger feedstock collectors and aggregators or there may also be some basic filtering and pre-processing to remove impurities such as water and pieces of food before the WFO is sold on for biodiesel production. Compared to other biofuels, the WFO to biodiesel supply chain is most typically characterised by a large number of relatively small feedstock ‘producers’ with a local collection infrastructure. Several companies are also investing in exploring additional novel sources of waste oils and fats, for example retrieval of oils from food waste or of waste fats from the sewerage system, but these sources require investment in research and development and modifications to plant which, companies indicate, remains difficult in the current investment climate (Toop et al., 2013).
One company that is active in this area is Brocklesby Ltd, who is involved in the collection and processing of food waste with high oil and fat content, such as foods like pies, sausage rolls, pastry and crisps. In 2013 Brocklesby commissioned an innovative processing facility that extracts oils and fats from the waste streams produced by food manufacturers and expect to be processing more than 1,000 tonnes per week (Toop et al., 2013).

Biodiesel production using vegetable exhaust oils
The potential of using waste cooking oil as feedstock has been appreciated and explored by many researchers around the world. Kaur Sodhi and his team studied the potential of waste cooking oil to be converted into energy. They demonstrated the feasibility of this idea and optimized the transesterification process by changing different parameters in order to identify the condition that could give the highest yield (i.e. 93.8% of conversion) (Sodhi et al., 2017). To make the oils suitable for transesterification, they were previously heated at 60°C and then filtered using the

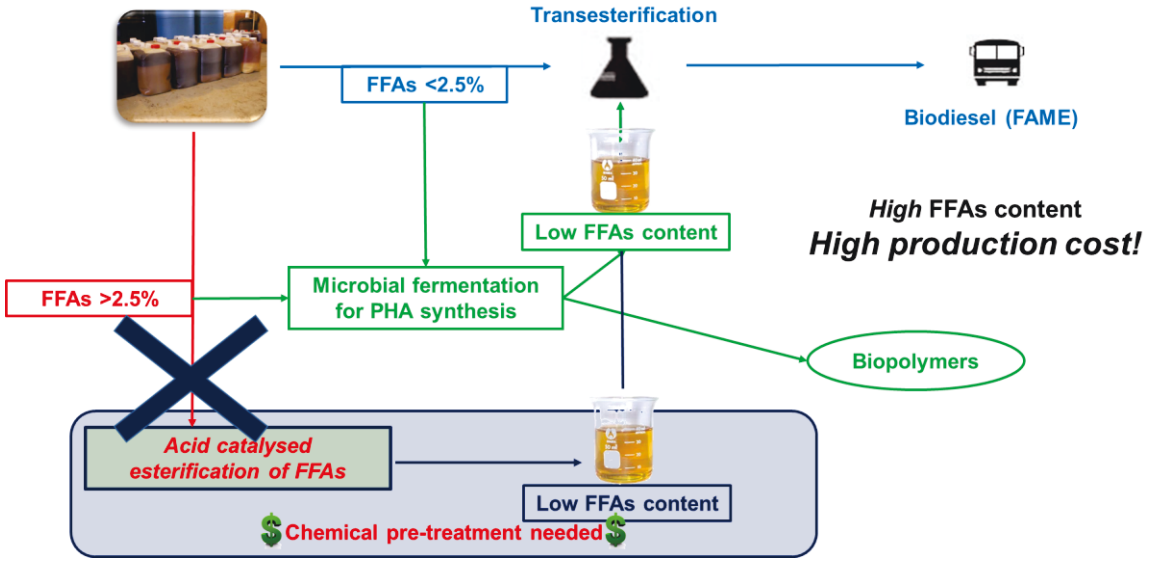
muslin cloth until the sample resulted as clear as possible and free from any sort of contaminants. Three different parameters, i.e. reaction temperature, catalyst concentration and alcohol-oil molar ratio, were optimized and the most favorable optimum condition that resulted in the maximum yield was at 60°C, with 1% of catalyst concentration and molar ratio 5:1, until the mixture was completely stabilized. The Open University of Sri Lanka also explored the same theme and even tried to fabricate a Small and Medium-sized Enterprises (SME) level plant for the production of biodiesel, from waste cooking oil. Waste cooking oils were collected from restaurants or domestic users and since their FFAs' content was too high to be directly converted into biodiesel (>2%) they were subjected to an acid-catalyzed esterification process. In this process sulfuric acid was used to decrease FFAs content and the reaction was conducted at 60°C and 2 hours of time, at 125 rpm (Lokuliyana et al., 2016).

WFO for polyhydroxyalkanoate(PHA) production

Among the possible routes for WFOs exploitation, alternatives to biodiesel production, a new emerging area is represented by its utilization as raw material for microbial fermentation. Several studies have already proposed the microbial conversion of fatty acids rich media in renewable fuels and chemicals (Dellomonaco et al., 2010; Jeon et al., 2012), suggesting that fatty acids could become a sustainable feedstock for industrial production (Doi et al., 2014; Sathesh and Lee, 2015). In this frame, investigation of possible sustainable production of biopolymer has been already carried out. In view of process sustainability, the use of waste materials as carbon sources represents a valuable alternative to reduce mcl-PHAs production costs. In particular, WFO are a promising source of related carbon source for mcl-PHA bioprocess. However, the heterogeneous and not-reproducible nature of these wastes, together with the complexity of the metabolic routes fuelling PHA precursors, make the achievement of a biopolymer with defined composition a challenging goal to be pursued. As a fact, despite the high production levels achieved, 20- 40% of cell dry weight, only polymers with mixed and variable compositions (from C8 to C16) have been produced from different *Pseudomonas species* fed with WFO as related C-sources (Haba et al., 2007; Gamal et al., 2013; Follonier et al. 2014).

The focus of this part of the thesis is the study of PHAs production from WFOs characterized by high FFAs content.

The use of unattractive FFAs rich WFOs as substrates for *Lipo* systems was expected to lead to an "improved" raw material (triglycerides) with lower free fatty acid content that could be directly addressed to biodiesel production (avoiding any pre-treatment and lowering the amount of requested alkaline catalyst), to achieve biopolyesters production.



Scheme of bioprocess for valorization of FFAs rich wastes in biodiesel-convertible substrates and biopolymers

The moving idea behind the studies is that microbial fermentation of WFO by properly designed strains, able to produce PHA from FFAs, but not secreting lipases, may turn out in a twofold advantage: allowing biopolymer production, and generating an "exhausted waste oil" with reduced FFAs content, thus more substantially exploitable for conversion in biodiesel (**Chapter 3.2**). Although, experiments conducted on properly designed *E. coli* strains, conferred the proof of concept with recombinant, extracellular lipase-free microorganism, the biopolymer yield was not satisfactory for industrial exploitation of the bioprocess. Further investigation regarded a native PHA producer microorganism and an its lipase-free mutant (**Chapter 3.3**). Results obtained in the following chapters lay the bases for the development of a circular bioprocess allowing PHA production from WFO as well as a more advantageous reuse of the "exhausted waste oil" for biodiesel production. The interesting achievement obtained in this field sustained the establishment of a start-up aimed to industrial transfer of the set-up bioprocess.

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3. Biorefinery for biomaterials: how to connect biodiesel industry and PHA production

Production of bioplastic from waste oils by recombinant Escherichia coli: a pit-stop in waste frying oil to biodiesel conversion race

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PRODUCTION OF BIOPLASTIC FROM WASTE OILS BY RECOMBINANT *Escherichia coli*: A PIT-STOP IN WASTE FRYING OIL TO BIO-DIESEL CONVERSION RACE

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Abstract

Waste frying oil (WFO) is a complex and heterogeneous waste collected mainly from restaurants and, to a lesser extent, from food processors and households. In the last decades, interest for this waste has deeply grown and a strong competition in grabbing this raw material has begun. About 90% of WFOs collected in Europe is recycled for biodiesel production, being the high free fatty acids (FFAs) concentration in WFOs one of the main drawback limiting the yield of the conversion process. In this study we proposed a pit stop in the WFO to biodiesel conversion race by introducing an upstream microbial fermentation step aimed at reducing the FFAs content through its conversion into biopolymers, i.e. Polyhydroxyalkanoates (PHA). A properly engineered *Escherichia coli* strain, able to produce PHA exclusively from FFAs, but not secreting lipases, was tested in this process. Recombinant production of a near P(HHx) homopolymer was achieved in a process requiring an aqueous pretreatment step aimed to reduce the content of non-lipid carbon sources, which compete with FFAs for microbial growth impairing the PHA production. A WFO with a halved FFAs content was recovered after fermentation, thus rendering it more attractive for further conversion in biodiesel. Despite the quite low production yield achieved at this stage, the idea of no-competitiveness behind the process was verified, leaving space to further strain improvement strategies to boost PHA yield.

Key words: biodiesel, circular process, polyhydroxyalkanoates, FFAs reduction, WFO

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1. Introduction

1.1. Waste frying oils (WFO): current uses and supply chain

According to the UK Environment Agency (Environment Agency, 2009), Waste cooking (frying) oils (WCO-WFO) are purified fats of plant or animal origin, which are liquid at room temperature. Like all fats, WFOs are a water-insoluble mixture of triacylglycerol (TGA), diacylglycerol (DGA), monoacylglicerol (MGA) and free fatty acid (FFA). Moreover, WFO are typical characterized by the presence of small pollutants as small pieces of fried food and a higher content of FFAs compared to unused equivalent (Spöttle et al., 2013).

WFO is a complex and heterogeneous waste collected from diverse food preparations. WFOs have been traditionally recycled in several processes including: *i)* animal feeding; *ii)* oleochemical industry; and *iii)* energy production (incineration and biodiesel). However, following BSE scandals in the early 2000s and the ABPR 1069/2009 regulation amended in 2013, which authorizes the use of animal fats of all categories for oleochemical products, the share of WFOs allocated for biodiesel industry has increased noteworthy. As fact, 90% of WFO in the EU-27 is allocated for biodiesel vs 10% for oleochemical industry in 2013 (Spöttle et al., 2013).

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The use of WFOs for biodiesel represents a sustainable outlet for the disposal of this problematic waste, with the potential to offer significant Green House Gas (GHG) savings in accordance with the target imposed by the EU Renewable Energy Directive 2009/28/EC.

WFOs addressed to biodiesel production are usually collected from a large number of relatively small feedstock producers and often undergo several stages of basic filtering and pre-processing to remove impurities such as water and food residues. The quality of WFOs is determined by its cleanliness, the level of FFAs and water content which are dependent on the type of fried foods, the frequency of replacing the cooking oil with fresh one and the nature of the vegetable oil used for cooking. The major technical challenge for biodiesel production from WFOs is linked to FFAs concentration (Chai et al., 2014). It has been claimed that, when the FFAs level exceeds 5%, during the alkaline trans-esterification process, undesirable formation of soap becomes substantial (Banani et al., 2015). Soap formation results in yield loss and increased difficulty in biodiesel/glycerol separation (Kulkarni et al., 2006; Ma et al., 1998). A two-step conversion process has been generally applied to address this issue. In this process, an acid-catalyzed methanol demanding esterification precedes the traditional alkaline-catalyzed trans-esterification in order to lower the FFAs content (Agnew et al., 2009; Javidialesaadi and Raeissi, 2013).

Due to its interesting potential, WFO has acquired a real market value in the past few years. For restaurants or food processors WFO is primarily a waste that needs to be properly disposed. However, WFO is becoming an attractive raw material that some collectors are starting to pay for to receive from catering premises, even if prices paid for WFO are reported to vary widely, 380-910 €/ton in Europe and about 150 €/ton in the USA, depending on the oil quality (Smith et al., 2013). In addition, an increasing number of WFO thefts have been reported in the EU, and also in the USA (Spöttle et al., 2013).

The WFO supply chain has not been highly regulated. Restaurants are the major source of WFO followed by food processors and households. The main differences in composition between wastes from catering premises and households mainly consists in the complexity of the oil (one type *vs* a mixture of different oils respectively), and the FFAs content. As regard to this latter parameter, higher and more variable values ranging from < 1% to > 40% have been reported for food industry derived wastes in comparison with homemade ones (Banani et al., 2015; Chai et al., 2014; Corro et al., 2016; Javidialesaadi and Raeissi, 2013).

1.2. New routes for WFO valorisation: microbial fermentation to biopolymer production

Among the possible routes for WFOs exploitation, a new emerging area is represented by its utilization as raw material for microbial fermentation. Several studies have already proposed the microbial conversion of fatty acids rich media in renewable fuels and chemicals (Dellomonaco et al., 2010; Fernández et al., 2015; Jeon et al., 2012), suggesting that fatty acids could become a sustainable feedstock for industrial production (Doi et al., 2014; Sathesh and Lee, 2015).

In this frame, biopolymers are among the high-added value products obtainable through microbial conversion of waste oils. Biopolymers market, as fact, is forecasted to a Compound annual growth rate (CAGR) over than 10 % reaching a market size of 4.55 billion Euros by 2021. This strongly increasing trend is due to not only by the petroleum prices fluctuations but especially by the worldwide stringent environmental regulations, forcing the companies to search for new strategies to reduce the carbon content in their products. Although some example of innovative materials have already seduced the customers (i.e. Bio-PET), the demand for eco-friendly plastics made from bio-based polymers is growing at a high rate. This is due to products such as PLA, PHA, and starch-based polymers finding increased applications thanks to their biodegradability (Comaniță et al., 2015; Research and Market, 2016).

Polyhydroxyalkanoates (PHAs) are polyesters, synthesized and stored as intracellular granules by numerous prokaryotes, which have attracted industrial attention as environmentally friendly and biodegradable alternatives to petroleum-based plastics. Two main groups of polymers can be classified depending on the number of carbons in the monomer units: short-chain length PHAs (scl-PHAs) having from 3 to 5 carbon atoms and medium-chain length PHAs (mcl-PHAs) having from 6 to 14 carbon atoms. Differences in repeating unit composition influence the physical properties of PHAs (Doi, 1990). scl-PHAs show thermoplastic material properties similar to polypropylene, while mcl-PHAs possess elastic material properties similar to rubber (Liu et al., 2011; Muhr et al., 2013). Due to their high elasticity and low crystallinity, mcl-PHAs polymers have emerged as suitable materials for novel applications in cosmetics, paint formulations, other coatings, medical devices and tissue engineering (Park and Lee, 2004).

Monomer composition of PHAs depends on the nature of the supplied carbon source and on the metabolic pathway fuelling precursors for PHAs biosynthesis (Tan et al., 2014) (Fig. 1). In view of process sustainability, the use of waste materials as carbon sources represents a valuable route to reduce mcl-PHAs production costs. However, the heterogeneous and not-reproducible nature of these wastes, together with the complexity of the metabolic routes fuelling PHA precursors, make the achievement of a biopolymer with defined composition a challenging goal to be pursued. As a fact, despite the high production levels achieved, 20-40% of cell dry weight (CDW), only polymers with

mixed and variable compositions (from C8 to C16) have been produced from different *Pseudomonas* species fed with WFO as related C-sources (Cruz et al., 2016; Fernandéz et al., 2005; Follonier et al., 2014; Haba et al., 2007; Song et al., 2008).

Examples of PHAs production from fats rich waste raw material have been also reported for wild type and mutant variants of *Ralstonia eutropha* (Cruz et al., 2016; Riedel et al., 2014). In this case, the high production yields achieved (up to 100g/L) are linked to the production of P(3HB) or copolymer with only small amount of mcl monomers. Metabolic engineering strategies have been also applied to *Pseudomonas* species (Chung et al., 2011; Wang et al., 2011) as well as to *Escherichia coli* recombinant strains (Davis et al., 2008; Tappel et al., 2012), to achieve the production of mcl-homopolymers and/or mixed copolymers. However, only the feeding with defined fatty acids has shown to allow constant polymer composition.

An example of recombinant system able to convert WFO from homemade preparation in mcl-PHA with constant composition has been previously reported by our research group (Vastano et al., 2015). In this *E. coli* recombinant strain (*sPha*), monomers for PHA synthesis are fuelled only through the activity of recombinant ketoacyl-CoA reductase (PhaB). The lack of any other enzymes involved in the PHA pathways described by Tan et al. (2014) (PhaA, PhaJ, PhaG, etc.) allows the biopolymer production only when free fatty acids are used as carbon source. In this strain, a strict control of mcl-PHA repeating unit composition has been achieved by the presence of biosynthetic enzymes endowed with peculiar selectivity towards polymer precursors. As a fact, a P(HHx) near homopolymer has been produced by the developed recombinant system fed with different fatty acids in pure free form and also in complex matrices as homemade WFO preparations.

In this work, we aim to study the exportability of the above mentioned recombinant system on WFO deriving from catering services, characterized by high FFAs content. Different strategies have been tested for WFO valorisation into a high-added value product (mcl-PHA), including strain improvement and waste pretreatment. The idea behind the work is that microbial fermentation of WFO by properly designed strain, able to produce PHA from FFAs, but not secreting lipases, may turn out in a twofold advantage: allowing biopolymer production, and generating an "exhausted waste oil" with reduced FFAs content, thus more sustainingly exploitable for conversion in biodiesel.

2. Material and methods

2.1. Bacterial strains and growth conditions

Bacillus cereus 6E/2 was provided by University of Naples, Italy, culture collection. *Escherichia coli* Top 10 (Life Technologies, Monza, Italy) was used as host for gene cloning. *Escherichia coli* BL21 (DE3) (Novagen, Germany) was chosen as host for recombinant protein production (Table 1).

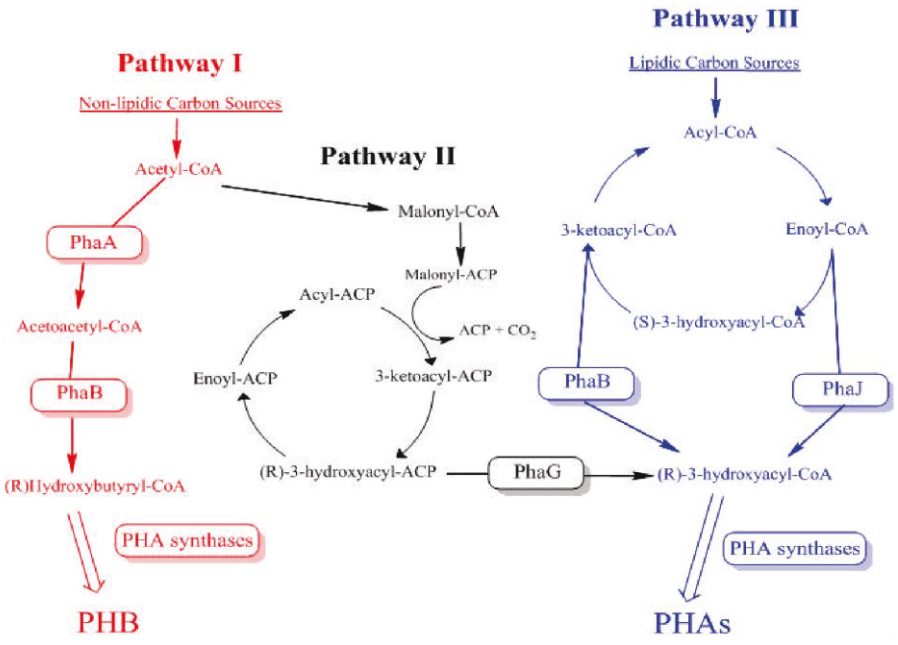

Fig. 1. The most representative metabolic pathways for PHA biosynthesis: Pathway I leads to the biosynthesis of PHB polymers by furnishing 3-hydroxybutyryl-CoA precursors from metabolism of non-lipidic carbon sources; Pathway II and III supply precursors for mcl-PHAs by channeling intermediates from fatty acid synthesis and degradation metabolisms respectively. The key enzymes responsible for diverting precursors towards polymer biosynthesis are highlighted. PhaA: β-ketothiolase; PhaB: ketoacyl-CoA reductase; PhaC: PHA synthases; PhaG: 3-hydroxyacyl ACP-CoA transacylase; PhaJ: enoyl-CoA hydratase

Table 1. Plasmids and bacterial strains

Plasmid or strain	***Relevant characteristics***	***Source or reference***
Plasmids		
pET40	kan^r, P_{T7lac}, pBR322 origin	Novagen
pET16SPHA	pET16b derivative, *B. cereus* 6E/2 *sphaRBC*$_{Bc}$	Vastano et al.(2015)
pET40A	pET40 derivative, *B. cereus* 6E/2 *phaA*$_{Bc}$	This study
DUETTOASPHA	Dual promoter vector pET16b-pET40 derivative, *B. cereus* 6E/2 *sphaRBC*$_{Bc}$ and *phaA*$_{Bc}$	This study
Strains		
B. cereus 6E/2	Wild-type	
Escherichia coli Top10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Life technologies
E. coli BL21(DE3)	F$^-$ *ompT hsdS*$_B$ (r$_B^-$ m$_B^-$) *gal dcm* (DE3)	Novagen
sPha	*E. coli* BL21(DE3), pET16SPHA	Vastano et al.(2015)
A-sPha	*E. coli* BL21(DE3), DUETTOASPHA	This study

Table 2. Oligos sequences with highlighted restriction enzymes sites: NdeI, underlined; KpnI, double underlined; ***SphI***, bold

Oligonucleotide	***Sequence***
phaAfw	GCAATTACATATGAGAGAAGCTGTCATTGTTGCGGG
phaArev	CGTAAT**GCATGC**GGTACCTTATAGTAATTCAAACACTCCTGCTG
pET40-SphIfw	GGAATGGT**GCATGC**AAGGAGATGG

All bacterial strains were routinely grown in Luria-Bertani (LB) broth (Sambrook and Russell, 2011) at 37°C, supplemented with ampicillin (100 µg/mL) if transformed, unless otherwise specified. For PHA production, strains were grown in Minimal Medium (MM) broth boosted with 6 % v/v of WFOs (MM: 1g/L Bacto Tryptone, 1g/L Sodium Chloride, 0.5g/L Yeast Extract).

2.2. PHA production and analysis

For PHAs production, recombinant *E. coli* strains were cultured in 250 mL Erlemeyer flasks containing 50 mL sterile MM growth media. Flasks were incubated at 37°C on a rotary shaker at 250 rpm. 0.5 mM IPTG induction was performed at a defined time (5 h from the inoculum) due to the interference in optical density measurements caused by the turbidity of oil containing growth media. Effective induction of protein expression was verified by performing SDS-PAGE analyses according to standard methods (Ausubel et al., 1998).

For PHAs analysis, cells were harvested by centrifugation (11,120 rcf for 10 min), washed twice with hexane, and lyophilized. PHAs were converted to the corresponding monomer-esters by combining 1 mL of chloroform and 1 mL of 15% H_2SO_4 in methanol (v/v) with 2-5 mg of lyophilized cells in a 6 mL pyrex glass tube (Agnew et al., 2012). The mixture was heated at 100°C in a heat block for 3 h followed by neutralization with 2 mL of 100 mg/mL $NaHCO_3$ in water. The mixture was vortexed and centrifuged 797 rcf for 10min and the aqueous layer was removed by aspiration. The organic phase (1 µL) was analyzed using an Agilent Technologies 6850A gas chromatograph equipped with a mass spectrometer 5973N (Agilent Technologies Italia, Milan, Italy) and a Zebron ZB-5 Phenomenex, 30 x 0.25 mm i.d. column (He 1mL/min) (Phenomenex, Bologna, Italy). The injection was done in splitless mode with Tinjector at 230°C. The temperature programme used was as follows: 40°C → 240°C at 20°C /min, 240°C for 10 min. The MS was operated in scanning mode between 40 and 360 m/z. The identification of compounds was performed by comparison of mass spectra with standard ones. PHA contents (mg/L) were calculated by using calibration curves, which in turn were obtained by using either 3-hydroxybutyrate or 3-hydroxyhexanoate (Larodan Fine Chemicals, Sweden) supplemented with benzoic acid as internal standard. %PHAs was expressed as the ratio between produced PHAs (mg) and CDW (mg) of lyophilized cell material.

2.3. Construction of A-sPha strain

A PhaA coding gene (*phaA*$_{Bc}$) was amplified from *B. cereus* genomic DNA, extracted as previously described (Vastano et al., 2015). The oligonucleotide primers were designed on the basis of those reported by Davis and coauthors (Davis et al., 2008) for a *phaA* homologue (Table 2). Sequences for proper restriction sites were included in primer design for cloning in pET40 vector (Life Technologies, Monza, Italy).

The *promphaA*$_{Bc}$ sequence was then amplified from the cloning vector with the oligo pairs pET40-SphIfw/phaArev (Table 3), in order to add the T7 promoter sequence at the 5' extremity, and finally cloned in *SphI* digested pET16SPHA, obtaining DUETTOASPHA plasmid. Amplification was performed using High Fidelity Q5^R polymerase (New England Biolabs, Ipswich, Massachusetts, USA) according to manufacturer's instruction. All cloned PCR products were checked by sequencing (PRIMM Sequencing Service). Recombinant plasmids were transformed in *E. coli* strains using standard transformation methods (Sambrook and Russell, 2001).

2.4. WFOs suppliers

Yellow fluid (YF) WFO was collected by a local restaurant. Brown dense (BD) WFO has been supplied by FARGECO Srl, a regional service for waste oils collection from catering premises and food-factories.

2.5. Water extraction of WFOs

A defined amount of WFO was twice extracted with the same volume of room temperature water. The two phase solution was shacked until mixture results opaque. WFO-water dispersion was resolved by centrifugation (5,524 rcf for 10 min). Upper lipidic phase was collected, avoiding the white opalescence phase at interlayer, and a second step of extraction was undertaken. WFOs was autoclaved before use. Tap water, Demineralised water and Elix® water (Merck, s.p.a, Milan, Italy) have been tested in the extraction process.

2.6. FFAs calculation

Sample of 1mL of WFO was dissolved in 10mL of isopropanol with Phenolphthalein as pH indicator. %FFAs value was calculated according the Eq. (1) considering oleic acid as standard:

$$\%FFAs = (v - b) \times N / w \times 282 \times 100 \quad (1)$$

where: v is the volume of titration solution; b is the volume of the titrant for isopropanol neutralization; N is the Normality of the titration solution; w is the weight of the oil sample; *282* is the molecular weight of oleic acid (g/mol). NaOH 0.025N solution was used as titration solution.

3. Results and discussion

3.1. PHA production from industrial WFOs

Two WFOs, furnished by two restaurants, were tested as raw materials for mcl-PHAs production. Although their characterization was out of the scope of this work, the two oil variants were labelled as YF (Yellow Fluid preparation; 4.2 % FFAs) and BD (Brown Dense preparation; 8.5 % FFAs) on the base of their different colour and density. The *E. coli sPha* recombinant system, already proved to be able to produce P(HHx) homopolymer from several household cooking oil preparations (up to 5% cell dry weight) (Vastano et al., 2015), was tested for polymer production in Minimum Medium boosted with 6% of both WFOs. However, no PHAs production was achieved in these conditions. Of course, differences between homemade and catering oil preparations can affect the polymer yield, as already observed among different domestic preparations (Vastano et al., 2015). This is probably due to several and uncontrolled parameters such as time and temperature of cooking, initial oil composition, compounds originated by cooked foodstuffs, etc.. In particular, "deep frying", the typical cooking technique for catering fries, might have generated a waste with peculiar composition.

In spite of the absence of PHAs production, microbial growth was not hampered by the presence of WFOs. This behaviour suggests that, in addition to FFAs, these WFOs may contain a higher amount of alternative C-sources (carbohydrates and/or amino acids) compared to WFOs from household preparations. These preferred nutrients may be utilized instead of FFAs, preventing their fuelling towards PHAs metabolic route.

In order to verify this hypothesis and promote biopolymer production, two alternative approaches have been carried out: *1)* genetic improvement of the producing strain, aimed at supporting PHA biosynthesis from non lipidic C-sources; *2)* implementation of the raw material, including a pre-treatment step to extract undesired carbon sources. These strategies are not exclusive alternatives allowing the production of different kind of biopolymers. As fact, according to the metabolic routes involved in the process (Fig. 1), the first approach may promote the conversion of WFOs into scl-mcl co-polymers (through Pathway 1 and 3), the second one, instead would produce a mcl-PHAs near-homopolymer (through Pathway 3).

3.2 Approach 1: engineering sPha strain for conversion of untreated WFOs

A new system allowing PHAs production from carbohydrate carbon sources, named *A-sPha,* was constructed. In addition to the *phaRBC* operon, this system expresses a β-ketothiolase coding gene from *B. cereus* (*phaA*$_{B.c.}$), whose product has been shown to be involved in the production of P(3HB) when expressed in *E. coli*. Considering the metabolic routes activated in the new engineered strain, a higher 3HB fraction is expected to be incorporated in the produced polymer with respect to *sPha* (Fig. 1).

The productive performances of *A-sPha* were tested in MM with each WFOs variant. However, as for *sPHA*, no PHA production was observed when the system was fed with YF or BD. The poor efficiency of PhaB$_{B.c.}$ in reducing acetoacetyl-CoA generated by PhaA$_{B.c}$ from pathway 1, may explain the absence of differences in *sPha* and *A-sPha* behaviour when fed with WFOs. As a fact, a high specificity towards the incorporation of 3-ketoacids with 6 carbon atoms into the growing polymer has been previously suggested for PhaB from *B.cereus* (PhaB$_{B.c.}$).

3.3. Approach 2: water extraction of the raw material

With the aim to remove the non-lipidic carbon sources from the waste, a pre-treatment step of WFOs consisting of an aqueous extraction, has been performed. Three kinds of water, with different pH

and salts composition, were tested for the extraction step (Table 3) to assay the effects of these parameters in solubilizing non-lipidic compounds from WFOs.

Table 3. Properties of extraction waters

Water	*pH*	*Conductivity [µS/cm]*
Tap Water	8.3	696
Demineralised Water	7.1	28
Elix®Water	6.8	3

The pre-treated WFOs were added to MM medium and PHA production by *sPha* strain analyzed (Table 4).

Table 4. PHA production from *sPha* strain in the presence of WFO. ND, not detected; CDW, cell dry weight; PHA, polyhydroxyalkanoates; YF and BD, industrial waste frying oils preparations. The SD of each series of results was less than ±15%

WFOs	*Extraction Water*	*CDW [mg/L]*	*PHA amount [mg/L]*	*PHA [%]*
YF	Untreated	709.75	ND	ND
	Tap water	359.00	0.6	0.2
	Demineralized Water	377.63	0.6	0.2
	Elix®Water	362.75	1.4	0.4
BD	Untreated	466.25	ND	ND
	Tap water	409.87	3.5	0.8
	Demineralised Water	437.50	4.5	1.0
	Elix®Water	495.63	3.7	0.8

A reduction in microbial biomass was observed as a consequence of water extraction for YF WFO, independently of the water properties. This result is due to the removal of competitive nutrients. As expected, in this way the FFAs consumption was promoted, determining biopolymer production.

Comparing the biomass amount between the two untreated wastes, a lower CDW was obtained for BD WFO. The complexity and heterogeneity of these raw materials may explain this data. Moreover, in contrast with YF WFO, the water extraction treatment was effective in allowing PHA production but did not alter microbial growth.

No significant effect of the different extraction waters was observed in terms of CDW and PHA production. Up to 1% PHA production could be achieved after the pre-treatment using BD WFO. WFOs were easily recovered after microbial fermentation and characterized for the FFAs content. A FFAs reduction ranging of about 40 and 60% respectively for YF and BD WFOs has been verified.

Independently from WFOs and extracting water, a P(3HHx) near-homopolymer, with a quantity of 3-hydroxybutyrate not exceeding 1%, was produced. Despite the low level of accumulated biopolymer, this is the first reported example of an *ad hoc* designed recombinant strain able to produce an almost homopolymeric mcl-PHA from industrial WFOs. These results demonstrate the effectiveness of the pretreatment step in generating a raw material deprived of those competing C-sources (mainly carbohydrates and amino acids) that may have prevented the triggering of PHA synthesis in the untreated WFOs.

3.4. Design of a circular process for WFO valorization

The introduction of an upstream microbial fermentation step in the WFO to biodiesel conversion route was found to satisfy a two-fold purpose: converting the WFO into a high-added value mcl-PHA, and generating an "exhausted waste oil" more suitable for biodiesel production. The choice of a non-lipase secreting microorganism allowed to generate a waste with reduced FFAs without impairing tryacylglycerol content, with a potential advantage in terms of trans-esterification yield. Despite the lower PHA production level achieved in comparison with native biopolymer producers, such as *R. eutropha* or *Pseudomonas* species (Lee et al., 2012; Riedel et al., 2012; Riedel et al., 2014), the idea of no-competitiveness with respect to WFO conversion in biodiesel has been verified.

In addition, being potentially rich in soluble carbohydrates and proteins, the extraction water could be used as growing medium in alternative to MM medium tested in this study. Although only preliminary experiments have been carried out in this frame, extraction water did not hamper microbial growth and in some specific conditions, it allowed biopolymer production (data not shown). In Fig. 2 a possible circular process for WFOs valorisation is depicted. According to the idea of zero-waste process, a pyrolysis of residual biomass after biopolymer extraction has been proposed for production of bio-oil and bio-chair (Wei et al., 2015).

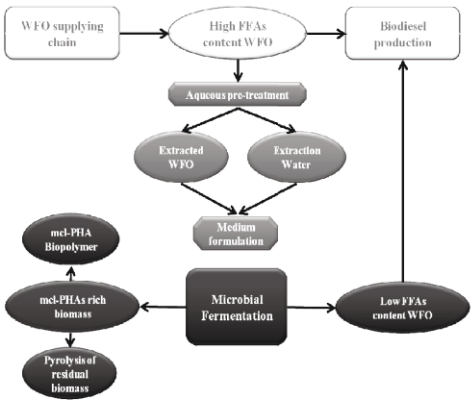

Fig. 2. Proposed circular process for WFOs valorization

4. Conclusions

In this study, a recombinant *E. coli* strain was tested for PHA production on two different catering oil preparations. Two different strategies were tested for WFO valorisation, including strain improvement

and waste pretreatment. The two approaches would potentially result in the valorisation of WFOs into two kind of biopolymers differing in their monomeric composition.

Biopolymer production was achieved only after a WFO pretreatment step, which was helpful to remove most of the competing C-sources for microbial growth. Engineering the *E. coli* genetic makeup introducing a PhaA coding gene, did not turn out into polymer production and is a point that needs to be further investigated. Replacing the *B. cereus* -ketoreductase PhaB, endowed with a peculiar specificity towards C 6 monomeric precursors from fatty acid metabolism with a homologue able to act also on keto intermediate with 4 carbon atoms, could promote polymer production even in the presence of non lipidic carbon sources. The study also lays the bases for the development of a circular process allowing PHA production from WFO as well as a more advantageous reuse of the "exhausted waste oil" for biodiesel production. Optimization of polymer production yield can be achieved by further improvement of the recombinant *E. coli* genetic background, or in alternative by testing native producers after carefully knock-out of their lipase coding genes.

In conclusion, validation of the proposed process will require a strict setting up of all the process steps. Optimization of the water extraction protocol, the verification of the use of the extracted water as bacterial growth medium, as well as the effect on the transesterification process from the "exhausted waste oil" will be the key points to be further evaluated in terms of the overall economicity of the process.

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3. Biorefinery for biomaterials: how to connect biodiesel industry and PHA production

Exploitation of recombinant and native PHA producers in designed Bioprocess

3.3 Exploitation of recombinant and native PHA producers in designed Bioprocess

Engineered recombinant PHA producers

The idea presented in the Chapter 3.2 aimed to the development of a new sustainable bioprocess was applied to the already characterized *LipoA* and the improved system *LipoB* (Chapter 2.2) with a systematic approach on WFOs with characteristics more similar to whose of target waste. Firstly, the effect of free fatty acids concentration on the expression of the heterologous proteins involved in PHA biosynthesis as well as their toxicity effect on microbial growth, was investigated. Preliminary experiments with oil preparations mimicking WFOs (mimetic WFO, mWFOs) were carried out in MM medium to assess the feasibility of the proposed process. mWFOs were prepared doping corn oil with a standard FFA (oleic acid) at three different concentrations (w/w): 10, 20 and 50% (**Table 3.3.1**). Both *LipoA* and *LipoB* were tested with these mWFOs.

Strain	Medium	Doped oil	cdw mg/L
Lipo A	MM	mWFO 10%	588
		mWFO 20%	433
		mWFO 50%	560
Lipo B	MM	mWFO 10%	635
		mWFO 20%	1252
		mWFO 50%	615

***Table 3.3.1**: Microbial growth with different mWFOs; cdw, cell dry weight (standard deviation is under 15%).*

Results indicate that a concentration of free fatty acids up to 50% in oil does not interfere with the microbial growth as well as with the expression of the enzymes involved in PHA production.

Microbial fermentation in the presence of WFOs

Further experiments were conducted using waste frying oils. The main differences between wastes from the different sources concern: i) nature of the oil (peanut oil, olive oil, corn oil etc.), ii) presence of different amount of water, iii) presence of food particles, iv) content of free fatty acids and v) density. The experiments were carried out employing households (WFO-A) and industrial (food services facilities) waste oils (WFO-B) (**Table 3.3.6** in Materials and Methods).

A second basal medium (SB), in place of the minimal medium MM, was also tested in order to obtain more reproducible conditions assured by the buffering salts. Data demonstrate that in all the tested conditions, except for WFO-A1, there was a clear biomass accumulation and over expression of the recombinant proteins. The absence of microbial growth in WFO-A1 may be due to the presence of toxic agents generated by the heat treatment (Dostálová et al., 2005).

As shown in **Table 3.3.2**, cell growth in SB conditions is not different among the two used strains, whereas there are huge differences between the two strains (*LipoA* and *LipoB*) when grown in MM. When MM was used, growth of *LipoB* was at least 2-fold

higher than *LipoA* for each WFOs. On the other hand, in the presence of SB both systems revealed similar biomass accumulation always higher than 1 g/L. In order to evaluate the effectiveness of the approach for WFOs pre-treatment for all conditions, FFAs content was measured after microbial fermentation. These analyses confirm that SB is the best culture medium not only as regard to microbial growth but also in term of reduction of acid content of WFOs. As fact MM conditions display a range of reduction between 70 and 30 %, while in case of SB the performance is higher than 50% in all tested conditions. Taking into account the composition of initial WFOs (**Table 3.3.6** in Materials and Methods), all these results suggest that there is a correspondence between the cellular growth and the consumption of free fatty acids in the medium. Since the promising results observed in SB, the PHA production was analysed for both systems with all three WFOs in this medium. GC-MS analysis reveal a high variability in terms of polymer yield. Moreover, only for *LipoB* with B2 PHA production higher than 100 mg/L was observed (mg PHA / mg cdw about 10%).

Strain	Medium	WFO	FFAs reduction %	cdw [mg/L]
Lipo A	MM	A2	69	382
		B1	33	486
		B2	60	407
	SB	**A2**	**84**	**1,300**
		B1	**69**	**1,292**
		B2	**64**	**1,636**
Lipo B	MM	A2	40	809
		B1	58	870
		B2	44	1,157
	SB	**A2**	**79**	**1,035**
		B1	**56**	**1,115**
		B2	**76**	**1,665**

Table 3.3.2*: Microbial growth with different WFOs; FFA reduction % estimated by the value of unfermented oils reported in Table1, cdw, cell dry weight (standard deviation is under 15%).*

Transesterification of fermented WFOs: Bioprocess proof of concept

The idea underlying the project has been verified by transesterification of oils characterized by high (*un-fermented*) and low (*fermented*) FFAs content. Results demonstrate the efficacy of the proposed pre-treatment. As a fact, the high content of free fatty acids of *un-fermented* oil has deleterious effects on the transesterification reaction, while in case of *fermented* (*treated*) WFOs (*tr*WFO) the reaction clearly occurred. Figure reported below regards the conversions of B1 and B2, before and after fermentation in SB (**Figure 3.3.1**).

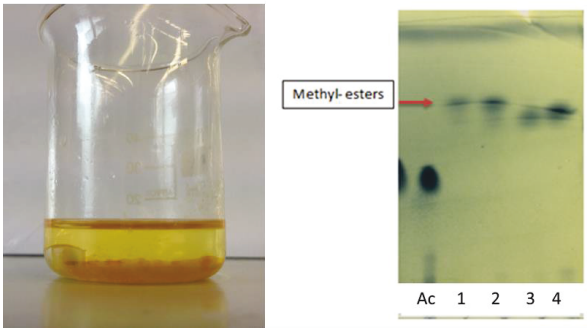

***Figure 3.3.1**: Left: transesterification of trB1, glycerol perfectly separated at the bottom of the becher; Right: TLC of oils: Ac, oleic acid; 1) trB1 (after fermentation); 2) trB2 (after fermentation); 3) B1 un-fermentation; 4) B2 un-fermentation.*

The TLC (**Figure 3.3.1 right**) shows the presence of a band corresponding to methyl esters, higher than the one of triacylglycerol (Leung et al., 2006), only in 1 and 2 conditions. As fact, for B1 and -B2 no bands appear after the reaction, on the contrary transesterification of *tr*B1 and *tr*B2 allow TAG conversion in biodiesel (FAME).

Although, experiments conducted on properly designed *E. coli* strains, conferred the proof of concept with recombinant, extracellular lipase-free microorganism, the biopolymer yield was not satisfactory for industrial exploitation of the bioprocess. Further investigation regarded a native PHA producer microorganism and an its lipase-free mutant.

Switch to native PHA producers

Due to the dual nature of the strategy, the microorganism chosen for this purpose has been selected among the major PHAs' producers and several considerations have led to the selection of a *Pseudomonas resinovorans* strain. *P. resinovorans* NRRLB-2649 is the microorganism chosen for the pre-treatment of waste frying oils (**Figure 3.3.2**). This choice has mainly been driven by its high PHAs producing abilities, from related and non-related C-sources. Moreover, the selected *P. resinovorans* is characterized by the presence of only a gene (*lip*) coding for extracellular lipase which can hydrolyze TAGs of WFO wrecking its quality for biodiesel conversion. The influence that lipase secretion has on the PHAs production and FFAs reduction has been investigated by the construction of the *lip* gene knock-out mutant.

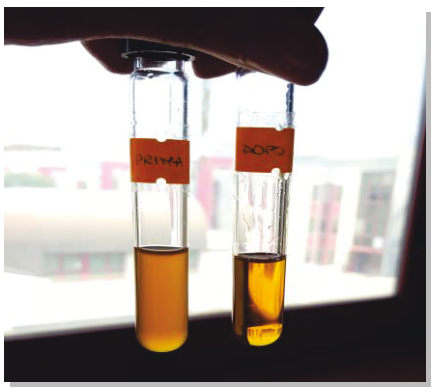

***Figure 3.3.2**: In the picture oil, before and after the pretreatment, is shown*

Comparison between wt and lip⁻

To evaluate how lipase's expression affects the pre-treatment purposes we compared both systems to define which one is the best in terms of FFAs reduction and PHAs' production. PHA production and FFAs reduction were analyzed at 24 h, 48 h and 72 h of fermentation in Medium E. The culture was supplied with WFO characterized by FFAs content of 10.5 %. As expected by the high acid content, the un-treated WFO was not successfully transesterificated, revealing a biodiesel conversion less than 5% when transesterificated with 1% of NaOH.

Data reported in **Table 3.3.3** demonstrate the absence of significant difference between *lip⁻* and *wt*, in terms of the purposes of our interest: PHAs production and FFAs reduction (with the exception of the 24 h values). PHAs production obtained with both *P. resinovorans* strains is similar to the one reported by Cruz which used uncharacterized waste cooking oil as a carbon source for PHA production (Cruz et al., 2015) Moreover, values obtained at 72 h are quite close to the ones reported by Ashby et al. which have conducted experiments under two-stage fermentation conditions, to maximize biomass production in the first phase and PHAs production in the second one. As fact, after 24 h plus 48 h, values ranged between 1.3 and 1.9 g/L and the higher yield was given by the pure substrates coconut oil and oleic acid (Ashby et al., 1998).

After 48 h of fermentation, FFAs content of the recovered oil is low enough to guarantee an acceptable biodiesel conversion (yield around 70-80%) if transesterifications were performed with 1% NaOH. However, changes in reaction protocol highlighted the variance related to different FFA reduction. As fact, the oil derived from *lip⁻* fermentation retained the same biodiesel yield when the amount of used catalyst was reduced up to 20% (final concentration of NaOH 0.8%). In contrast, *wt* derived oil treated with the same modified protocol revealed a biodiesel yield no higher than 50%. Reducing the amount of the base catalyst could give huge benefits in terms of biodiesel production's costs and in terms of disposal's costs of the alkaline solution.

	PHA (mg/L)		**cdw [g/L]**		**% FFA**		**% Biodiesel conversion**	
	lip⁻	*wt*	*lip⁻*	*wt*	*lip⁻*	*wt*	*lip⁻*	*wt*
24 h	148	160	1.66	1.51	7	8	49	3
48 h	582	522	2.49	2.63	6	7	67	65
72 h	1334	1354	3.80	3.92	4	5	79	72

***Table 3.3.3**: %FFA, %Biodiesel conversion and mg PHA extracted values (standard deviation is under 20%) related to P.resinovorans wt and the mutated strain, grown on Medium E and Waste Frying Oil. Transesterification was carried out with 1% of NaOH.*

Bioprocess Optimization

Different concentrations of glycerol were tested as boosts of biopolymer production. PHAs' accumulation is triggered by imbalances in nutrient conditions, such as excess carbon source and limited nitrogen availability. Therefore, varying the source and concentration of the carbon source added it is possible to appreciate variation in the PHA's production. Glycerol has been chosen as an additional carbon source since is the predominant by-product of biodiesel production. Therefore, its reuse as an additional carbon source in the medium defined above could represent an alternative to its disposal, contributing also in reducing biopolymer's overall costs since a waste sub-product is used as substrate for its production (Phithakrotchanakoon et al., 2015).

Two glycerol concentrations 0.2% v/v and 0.8% v/v were tested: the first one was the most reported in literature for the production of PHAs in *Pseudomonas*, while the second represents the concentration which assures a complete reintroduction of glycerol, derived from the transesterification step, in a circular process avoiding any sort of its disposal. This value was calculated basing on: i) 95% biodiesel yield ii) 20% of oil volume reduction after fermentation (i.e.: First fermentation: 1 L of medium E + 120 mL of WFO→ collection of ≈100 mL of WFO → transesterification of ≈100 mL of oil with 95 % of yield = biodiesel + 8 g of glycerol → Second fermentation: 1 L of medium E + 8 g of glycerol (0.8%) + 120 mL of WFO).

A	**PHA [mg/L]**		**cdw [g/L]**		***% FFA***		**% Biodiesel conversion**	
0.2% glycerol	*lip⁻*	*wt*	*lip⁻*	*wt*	*lip⁻*	*wt*	*lip⁻*	*wt*
24 h	161	205	1.35	1.45	*9*	*9*	45	10
48 h	593	756	2.30	2.69	*7*	*7*	77	75
72 h	1,547	1,530	3.62	4.11	*5*	*6*	79	80

B	**PHA [mg/L]**		**cdw [g/L]**		**% FFA**		**% Biodiesel conversion**	
0.8% glycerol	*lip⁻*	*wt*	*lip⁻*	*wt*	*lip⁻*	*wt*	*lip⁻*	*wt*
24 h	179	135	1.22	1.46	9	9	40	10
48 h	833	680	3.11	2.81	7	7	71	75
72 h	1,499	1,234	4.22	3.97	5	6	78	77

***Tables 3.3.4 A-B**: Results of lip⁻ and wt, grown on media containing 0.2% (**A**) and 0.8% (**B**) of glycerol respectively (standard deviation is under 15%).*

0.2% of glycerol implements PHAs' production with an increase close to the 15% when compared to the condition glycerol-free. However, this increase does not get any higher when the higher glycerol concentration is used. In fact, the concentration of 0.8% produces a quantity of PHAs comparable to the one obtained when 0.2% glycerol is used. Biodiesel conversion values and the one related to FFAs' reduction, instead, weren't much different from culture where WFO represent the only carbon source (**Tables 3.3.4 A-B**).

Double-fermentation strategy

At the end of fermentation, both systems were able to roughly halve oil's FFAs content. To further push FFAs' reduction and implement oil quality, a "second step" has been performed. After 72 h of fermentation (I step) cells were collected to analyze PHAs production while oils were recovered and used as a carbon source for a new 72 h fermentation (II step), for a total of 144h for bioprocess.

	% FFA	**Biodiesel Yield**	**CDW [g/L]**	**mg/L PHA**
lip^-_{Avg1-4}	<1	83%*	2.12	612
wt_1	5	79%	5.35	2,129
wt_2	3	77%	4.25	1,275
wt_3	15	4%	5.72	2,763
wt_4	16	5%	5.25	2,504

***Table 3.3.5**: Cell dry weigh, %FFA, % Biodiesel conversion and PHA production for II step wt and lip⁻ (standard deviation is under 15%). *Transesterification was performed using 0.5% of NaOH.*

All the experiments were performed in quadruplicate. *lip⁻* strain data revealed a satisfactory standard deviation lower than 15%, while the *wt* ones showed a high

variability which force to report data for each single experiment (**Table 3.3.5**). After the II step *lip*$^-$ shows a greater FFAs reduction than that achieved during the first 72 h (FFA: 4%), leading to FFAs content always lower than 1%. In contrast, *wt* shows FFAs content lower than that of I step only for one condition (wt_2). Moreover, in two trials (wt_3, wt_4) FFAs content reached values higher than that the initial oil. As expected, systems showed strong differences also in biodiesel conversion's yield: the yield is lower for *wt* with respect to *lip*$^-$ recovered oils. It is worth of note that *lip*$^-$ oils retain yields over 80% also when transesterificated with 0.5% of catalyst. This divergent behavior might be due to the expression of extracellular lipase in the *wt*. When the fermentation is carried out for 144 h, FFAs concentration decreases until it may overcome the "threshold" turning on lipase's expression. The activation of this extracellular enzymatic activity starts the consumption of TAGs that have been unused until then. This mechanism and the multiplicity of factors determining FFAs consumption/production for *wt* strain may explain the high variability observed for *wt* double fermentation experiments which was not observed for *lip*$^-$. Based on data reported in table xx, it is possible to hypothesize that the ascribed threshold is close to 3%. As fact, results in terms of PHAs production observed for wt_1 experiment (FFAs: 5%, PHA: 2,129 mg/L) may be caused by the conversion of hydrolyzed TAGs into biopolymers, while low acid content and biopolymers production of wt_2 may be related to the substrate shortage and to the absence of lipase expression (FFAs: 3%, PHA 1,275 mg/L). These effects are even more evident in wt_3 and wt_4 for which an early triggered lipase expression induced the release of high amount of carbon source (FFAs: 15%) resulting in high biopolymer production (PHAs: >2,500 mg/L). Although the FFAs reduction results clearly claim the *lip*$^-$ mutant as the best choice for pretreatment of acid WFOs, the PHAs production may challenge the choice of most suite microorganism for designed bioprocess. As fact, *lip*$^-$ obtains an optimum value for the FFAs' reduction, achieving a very high quality of the pre-treated oil, but with low PHAs' production (PHA: 612 mg/L). The carbon source that have supported *lip*$^-$ during I step finishes and there is no more to sustain PHA production during II step. In order to optimize the double-fermentation strategy, culture medium has been modified by the addition of an extra carbon source for growth supporting (*lip*$^-$). Glycerol has been selected as sustainable complementary C-source and it has been added in I step and/or II step media avoiding the lack of substrates determining decrease in PHAs production. It is also going to be studied the effect of glycerol on lipase induction and FFAs reduction for *wt*. This pool of experiments is currently ongoing and will be added in the final version of this PhD thesis.

Conclusions

Validation of the previous described bioprocess on native PHA-producer has been achieved in this part of thesis. In order, to design the best system for FFAs conversion in PHAs, genetic modification of the chosen *Pseudomonas* strain was performed. The effect of extracellular lipase activity on PHAs producing and FFAs reducing abilities was investigated. Both features appeared very similar between the two systems and no significative differences where observed in yield in biodiesel when oils were transesterificated using 1% NaOH as catalyst. However, when economic and environmental improvements were made (reduction of catalyst amount), biofuels production resulted more convenient for *lip*$^-$ based bioprocess. In order to push the PHA production glycerol was supplied as additional carbon sources and both strains responded with an increment of biopolymer production close to the

15% (0.2% glycerol). Substantial differences between the two systems were observed by prolonged fermentation. The oil resulting from *lip*$^-$ double fermentation had extremely low FFAs content when compared to the one *wt*. However, absence of lipase activity revealed the drawback of low PHAs production due to the scarcity of nutrients availability. The two tested systems have shown peculiar characteristic which make them alternative applicable depends on desired target: i) *lip*$^-$ should be selected if the preferred goal of bioprocess look at the pre-treatment step (FFAs reduction) enlarging the pool of biodiesel convertible substrates to low quality WFOs, ii) *wt* should be selected if biopolymers production represents the main purpose to be followed.
Further studies will be focused on the study of role of additional carbon source on polymer composition characterizing the chemical physical and mechanical properties of produced PHAs. Investigation and parameter optimization will lead to the set-up of custom bioprocess able to achieve satisfactory results for both goals.

Materials and Methods

Strains

LipoA, LipoB (Vastano et al. 2017). *Pseudomonas resinovorans* NRL B-2649 was obtained from NCAUR-ARS-USDA (Microbial culture Collection, Peoria, IL, USA). To prepare the *lip*$^-$ gene knock-out *P. resinovorans*, EZ-Tn5<KAN-2> transposon insertion reaction was performed with recombinant plasmid pETB1-lip provided by Daniel K. Y. Solaiman from the U.S. Department of Agriculture (Lee et al., 2012). To map the site of the transposon insertion, a chromosomal DNA fragment with the transposon-interrupted *lip* gene was amplified by PCR, using Taq polymerase and primers of TransFwd and TransRvs. The larger dimensions of the *lip*$^-$amplified fragment than the *wt* one suggested the success of the knock-out, which was further confirmed by DNA sequencing.

Culture Media

LB and MM see Vastano et al. 2015 M9* see Vastano et al. 2017. Medium E composition for 1L: $(NH_4)_2HPO_4$, 1.1 g; K_2HPO_4, 5.8 g; KH_2PO_4, 3.7 g. The pH was adjusted to 7.0 and the medium was autoclaved (Lee et al., 2012). 10 mL of a sterile 100 mM $MgSO_4$ solution and 1 mL of a microelement solution were added. The microelement solution contained the following (per liter of 1 M HCl): $FeSO_4 \cdot 7H_20$, 2.78 g; $MnCl_4 \cdot H_20$, 1.98 g; $CoSO_4 \cdot 7H_20$, 2.81 g; $CaCl_2 \cdot 2H_20$, 1.67 g; $CuCl_2 \cdot 2H_20$, 0.17 g; $ZnSO_4 \cdot 7H_20$, 0.29 g. Where reported glycerol was added. 0.8 % concentration able to completely reintroduce glycerol collected after transestification of fermented oil.

Mimetic Waste Frying Oils (mWFOs)

Mimetic Waste Frying Oils (mWFOs*)* were obtained doping corn oil with a standard FFA (oleic acid) at three different concentrations (w/w): 10, 20 and 50%.

WFOs

The *E. coli* experiments were carried out with two kinds of WFOs: households waste oils (WFO-A) and industrial (food services facilities) waste oils (WFO-B). Samples WFO-A0 and WFO-B0 represent the waste as they were at collection time. With the aim to obtain wastes with characteristics closer to those of low value used oils (Banani et al., 2015) both WFO-0 were subjected to different stressing processes. WFO-A1 and WFO-B1 underwent to a thermal treatment to catalyse the oxidation process which occurs during long cooking time; while the samples WFO-A2 and WFO-B2 represent the naturally aged oil after 5 months. In this way were obtained four derivate substrates (**Table 3.3.6**).

ID	Sample	% FFA	Density [Kg/m^3]
A0	WFO-A analysed soon after collection	0.4%	863
B0	WFO-B analysed soon after collection	1.0%	895
A1	WFO-A thermally abused (300°C, 16 hrs)	7.1%	859
A2	WFO-A naturally aged (5 months)	1.2%	894
B1	WFO-B thermally abused (300°C, 16 hrs)	3.4%	905
B2	WFO-B naturally aged (5 months)	5.1%	910

Table 3.3.6 *Properties of different WFOs preparation analysed.*

For *P. resinovorans* experiments WFO used as an additional carbon source was provided by Fargeco S.r.l (V. Scafatella - 80021 Afragola (NA) – Italia), which is specialized in the recovery of vegetable exhaust oils from restaurants, pubs, markets and any other place that produces exhaust oils during its activity. The oil has been stored for 10 months in uncontrolled conditions.

Free Fatty Acid content

The Free Fatty Acids (FFAs) content was estimated by standard titration with KOH 0.1% (w/v) as described in **Chapter 3.2**

Electroporation

Electroporation was performed as described by Lee et al. (Lee et al., 2012).

E. coli cultures in shaken flask

Recombinant *E. coli* cells from solid culture were inoculated in 20 ml LB medium in a 100 mL shaken flask. This preculture was grown over night at 37°C on a rotary shaker (200 rpm), then a volume of suspension sufficient to reach a final Optical Density (OD_{600}) of 0.1 was used to inoculate 250 mL shaken flasks containing 50 mL of each tested growth medium (MM+ 12% WFO, SB+ 12% WFO). 0.5 mM IPTG induction was performed at a defined time (5 h from the inoculum). Flasks were incubated at 37 °C on a rotary shaker at 250 rpm. In all the cases, effective induction of protein expression was verified by performing SDS-PAGE analyses according to standard methods.

P. resinovorans cultures in shaken flask

P. resinovorans cells were grown Medium E + 12% of WFO (v/v) at 30°C, with rotary shaking (250 rpm). Culture broth was prepared in 125 mL volumes in 250 mL flasks. The flasks were inoculated with a 1% inoculum from an overnight culture in LB. Kanamycin (Km, 50 µg/mL) was added only to LB media.

Transesterification

10 mL of oils was placed in a beaker equipped with a magnetic stirrer and a thermometer. Under agitation, the oil was heated up to a desired temperature (60 °C) on a heating plate. A fixed amount (2.8 mL) of prepared NaOH–Methanol solution was added into the oils, taking this moment as the starting time of the reaction. The operation was carried out similar to protocol described by Leung (Leung et al., 2006): 10 min at 1200 rpm followed by 10 min at 700 rpm. The products of reaction were allowed to settle overnight producing two distinct liquid phases: crude ester phase at the top and glycerol phase at the bottom. The reactions were performed at a molar ratio of 6:1 (methanol: oil) and with 1 % NaOH as catalyst.

Thin layer chromatography-TLC

The crude ester phase separated from the bottom glycerol phase was analysed by TLC on silica. A mixture of toluene–chloroform (7:3, v/v) was utilized as the mobile phase. Samples of biodiesel, acylglycerol and free fatty acids (oleic acid, OA) were normalized to 50 mg/mL in isopropanol. Two µL were then applied to the origin of a 10 × 10 cm chromatoplate. A solution of Phosphomolybdic acid (10% in Ethanol) was used as reagent, in order to activate the pigment for visualization it is necessary to heat TLC plates treated with this solution.

Biomass and oil Recovery

Once the cultures were stopped at the desired time, biomass was recovered through centrifugation of the liquid culture at 7500 rpm. After rinsing twice with an aqueous-isopropanol solution (1:1), followed by a 30-min centrifugation, the precipitate was collected, oven dried at 60°C over-night to remove any isopropanol residue, cooled at -20°C and lyophilized to dryness. Weights of the lyophilized cells constituted the cell dry weights. The supernatant was harvested to manually recover the oil suspended on the surface. An ulterior centrifugation of the oil recovered was performed at 8500 rpm to remove any liquid culture's residue. The sample was then stored at 4°C.

Biodiesel production assays

The yield in biodiesel was evaluated by NMR of transesterificated oils. ^{1}H NMR spectra were recorded on Bruker DRX-400 (^{1}H NMR: 400 MHz) in $CDCl_3$ (internal standard, for ^{1}H: $CHCl_3$ at d 7.26 ppm) Transesterification's yields were calculated directly from the area (A) of the selected signals:

$$Y\% = 100 \cdot (2 * A1 / 3 * A2)$$

where A1 and A2 are the areas of the methoxy (δ 3.6) and the methylene protons (δ 2.3), respectively (Gelbard et al., 1995). If necessary, samples were extracted with chloroform to remove any soap/glycerol residual.

PHA analysis

For *E. coli* cultures, PHA analysis have been performed by GC-MS as described in **Chapter 3.2**. For *wt* and *lip*$^-$ PHA's content was determined gravimetrically. The biopolymer was extracted by vigorously shaking dry cell pellets (100 mg), mixed with chloroform (30 mL) in a shaking flask overnight at room temperature, with constant stirring. After removing the cell debris by filtration through Whatman no.1

filter paper, the clear chloroform extract was placed at fume hood for solvent evaporation. The polymer was then re-dissolved in 5 mL of chloroform and precipitated using 10 volumes of methanol as a precipitating solvent. Polymer was weight until constant value and used for the determination of biopolymer production (%PHA).

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3. Biorefinery for biomaterials: how to connect biodiesel industry and PHA production

Crude glycerol from biodiesel industry and BEETOUT project

3.4 Crude glycerol from biodiesel industry and BEETOUT project

In frame of BEETOUT project following chapter focused on the development of new systems able to convert crude glycerol derived from biodiesel production into PHAs.
BEETOUT aims at setting a pre-competitive system to reduce waste of an industrial production, by creating a sustainable 2nd generation biomass value chain. More specifically, BEETOUT aims at improving the economic efficiency and environmental performance of the sugar industry by exploiting residues of the principal production chain to obtain biofuels and biomaterials, without sacrificing the energy balance of the process or the quality of the main product. BEETOUT focuses on currently less valuable residues compared to molasses, that is fresh and dehydrated sugar beet pulps. Exploitation of dehydrated sugar beet pulps is of great consequence as it is available in large quantities all year around, thus fitting industry needs regarding supplying consistency."
BEETOUT puts together a network of established research groups in agri-food, cell biology, biochemistry and industrial microbiology. More specifically, BEETOUT objective is ambitious and beyond state-of-the art. It aims to the market-oriented application of the concept of 2nd generation biorefinery to produce i) biodiesel from wet and dry sugar beet pulp thanks to oleaginous yeast fermentation and lipase catalysed transesterification and ii) PHAs from crude glycerol thanks to recombinant microorganism fermentation.Typically, biodiesel production generates about 10% (w/w) crude glycerol as the main by-product. Thus, approximately one pound of crude glycerol is generated when every gallon of biodiesel is produced. Due to the rapid growth of biodiesel industry, crude glycerol underwent a drastically fall in its price and is treated as pollutant waste that should be disposed of (da Silva et al., 2009).
Glycerol is a high-value and commercial chemical with thousands of uses, therefore the crude glycerol presents great opportunities for new applications. For that reason, more attention is being paid to the utilization of crude glycerol from biodiesel production to defray the production cost of biodiesel and to promote biodiesel industrialization on a large scale. The majority of crude glycerol is used as feedstock for production of other value-added chemicals, followed by animal feeds. In the last decade, the use of unwanted by-products as carbon source is even more explored in biotechnological processes, and glycerol has gaining attractions to become a very potential carbon source for PHAs biosynthesis (da Silva et al., 2009; Moralejo-Gárate et al., 2011). In particular, several studies reported that crude glycerol could be used to produce PHAs polymer (Ashby et al., 2011). The research on the feasibility of using crude glycerol for PHB production, with *Paracoccus denitrificans* and *Ralstonia eutropha* JMP 134, has shown that the resulting polymers are similar to those obtained from glucose (Mothes et al., 2007); also, some results obtained with recombinant *E. coli* demonstrate the production of PHA copolymers from crude glycerol (Phithakrotchanakoon et al., 2015). Therefore, PHAs production from glycerol represents a challenge for waste valorization and generation of high value-added products.

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3. Biorefinery for biomaterials: how to connect biodiesel industry and PHA production

Development of new systems for conversion of biodiesel by-product into tailor-made high added-value biomaterials

3.5 Development of new systems for conversion of biodiesel by-product into tailor made high added-value biomaterials

In order to enlarge the range of polymers produced and the pool of substrates convertible into PHA, *Omni* systems have been constructed. These systems are analogous to the *Lipo* ones, presented in **Chapter 3.1-3.2** but express also the β-ketothiolase PhaA, an enzyme able to activate the metabolic pathway from sugars, Pathway I (**Figure 1.2.1**). An additional interesting point is to verify if the differences, linked to regulation systems and metabolic background, observed in *Lipo* systems are also present in Omni systems.
Performances of these new strains were verified in two different media in order to selectively boost PHA production from Pathway I or Pathway III as a consequence of the culture medium used. For both media glycerol was selected as the growth supporting carbon source. *Omni* systems performances have been compared in a medium containing exclusively unrelated carbon sources (MYA) and in a medium containing both unrelated and related ones (SB*).
Since lipid carbon sources are absent in MYA broth, only the biosynthetic pathway from sugars (**Figure 1.2.1**) is active. Fermentation in SB* medium, instead, allows also the activation of the fatty acid β-oxidation pathway and potentially resulting in scl-mcl copolymers. SB* medium, as fact, derives from the M9 medium, which is already reported able to promote PHA production in *Lipo*- like systems (Chapter 2.2; Kihara et al., 2017). The basal carbon source for cell-growth (glucose) and the lipid carbon source for PHA biosynthesis (sodium octanoate) of M9 were substituted with glycerol and a mixture of long-chain fatty acids (C18 mix). This mixture is cheaper than sodium octanoate and similar to the lipid content characterizing crude glycerol. Testing PHA on this modified medium allows to close the gap to the industrial conditions achievable with the real biodiesel by-product. The efficiency of SB* medium was verified on *Lipo B*, which showed the same results obtained with glucose and sodium octanoate (data not shown).

PHA production fermentation
Results of fermentations in MYA reveal that, although there are not relevant differences in biomass among the three systems, PHA production from *Omni A* and *Omni B* is negligible, while that from *Omni C* is higher (12.6%) (**Table 3.5.1**). As regard to fermentations in SB*, despite the *Omni C* strain (LS5218) has a higher tolerance to fatty acids, there are no significative differences in biomass amount among all the strains (**Table 3.5.1**). Although the PHA production is very low for all tested systems, in contrast with what observed for *Lipo* analogous, *OmniC* reveals the comparable biopolymer accumulation to *OmniA* and *OmniB*. Considering the scarce production of PHAs obtained in almost all the analysed conditions, a metabolic engineering strategy was approached. Perez-Zabaleta *(*Perez-Zabaleta et al., 2016*)* demonstrated that NADPH plays a key role in PHAs production, since PhaB (β-ketoreductase) is a NADPH-dependent enzyme.

In order to increase the concentration of available NADPH, glutamate was chosen as additional nitrogen source to minimize NADPH consumption associated with the reductive amination of the α-ketoglutarate catalyzed by GDH (**Figure 3.4.1**). All the previously microbial fermentations were repeated adding glutamate (4.5 g/L) (**Table 3.5.1**).

Strain	MYA		MYA + glutamate		SB*		SB*+glutamate	
	cdw	%PHA	cdw	%PHA	cdw	%PHA	cdw	%PHA
Omni A	6.0	<1	6.4	<1	1.4	3.6	1.1	2.9
Omni B	6.0	<1	6.3	<1	1.4	3.4	1.4	2.9
Omni C	5.3	12.6	5.0	14.0	1.5	4.2	1.9	6.2

Table 3.5.1: *cdw and %PHAs produced by Lipo and Omni systems. N.A.= Not Analysed. The %PHAs columns are relative to a not purified extract. The standard deviations of each series of results was less than ± 15%.*

Results showed that glutamate addition does not affect PHA production from BL21 (DE3) systems (*Omni A* and *Omni B*). On the other hand, PHA production from *Omni C* is positively influenced by the glutamate addition in both the media, particularly in SB* where the production increment is close to 50%. This increment is more evident in SB*, since in this medium the different balance of carbon and nitrogen sources make the system more sensitive to glutamate addition. Crude PHA extracts were analyzed by ^{1}H NMR spectroscopy.

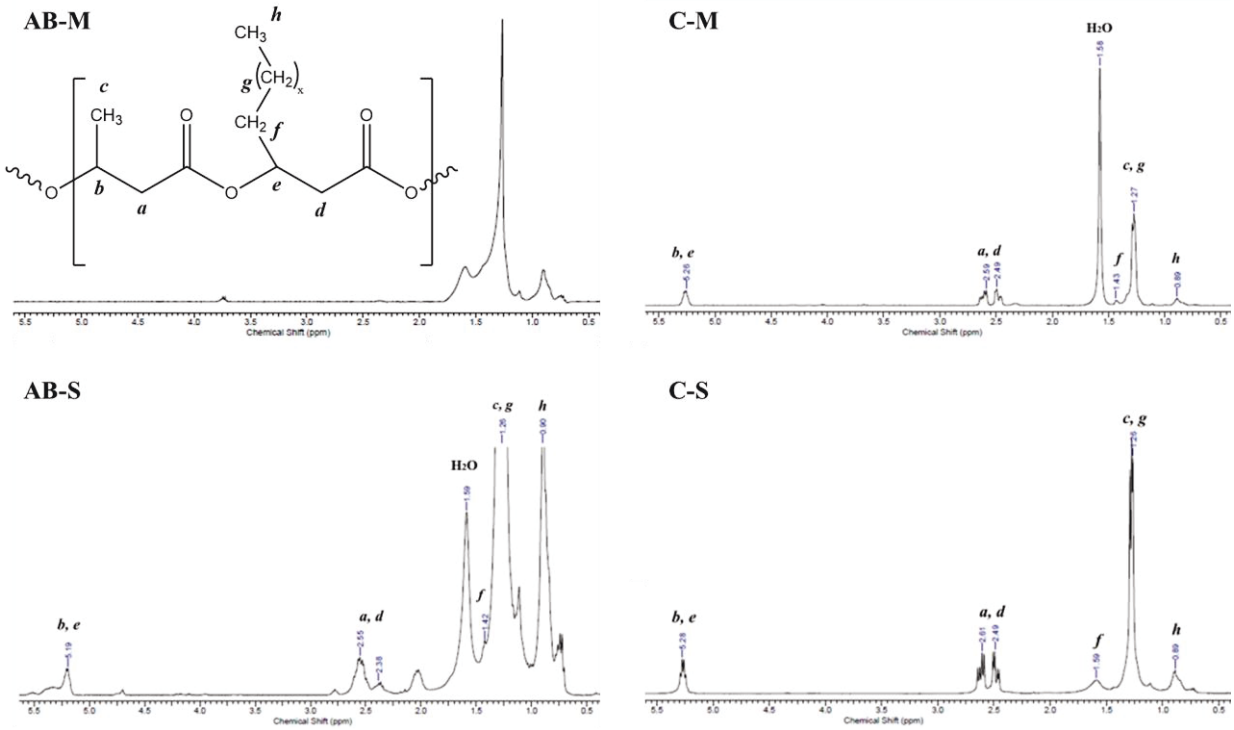

Figure 3.5.1: *^{1}H NMR spectra of PHA extract from OmniB (Representative also for Omni A system) and OmniC systems. AB-S) OmniB crude extract of SB* fermentations; AB-M) OmniB crude extract of MYA fermentations; C-S) OmniC crude extract of SB* fermentations; AB-M) OmniC crude extract of MYA fermentations. The general PHA structure is reported on the top: x= (0, n); x=0 - 3HV; x=1 – 3HHx.*

^{1}H NMR analysis revealed that *Omni A* and *Omni B* do not produce PHAs from unrelated carbon sources (**Figure 3.5.1; AB-M**). On the other hand, production of copolymer P(HB-HHx) has been observed from the related carbon sources. However, the low amount of produced polymer and the low grade of purity make difficult the determination of monomer composition (**Figure 3.5.1; AB-S**). ^{1}H NMR

analysis of *Omni C* crude extracts revealed the likely presence of co-monomers from fermentations both in MYA than SB* media (**Figure 3.5.1; C-M, C-S**), and in particular of 3HV moieties in MYA cultures and 3HHx in SB* cultures. This assumption is supported by the knowledge of the metabolic pathways activated by the supplied carbon sources and by the results previously obtained with *Lipo* systems. As a fact, MYA medium allows only the activation of the PHA biosynthetic pathway I, promoting the production of scl-PHAs; whereas the SB* medium enable also activation of the fatty acid β-oxidation pathway, supporting the production of scl-mcl-PHAs (**Figure 1.2.1**). Moreover, PhaA lacking *Lipo* systems are characterized by production of P(HB-HHx) whatever is the additional related carbon source (Chapter 2.1, Chapter 2.2). Further analyses through GC-MS are required to confirm these preliminary results.
Overall data claim that the β-ketothiolase selected for the construction of *Omni* systems, does not work properly as expected: in *Omni A* and *Omni B* (BL21 strain) the absence of biopolymers production from unrelated carbon sources (MYA) suggests that PhaA is not able to activate the biosynthetic pathway I. Moreover, its expression seems to interfere with polymer production also from related carbon sources (SB*). As a fact, a lower production has been achieved if compared with the respective *Lipo* systems. This disturbance hypothesis could also explain why the glutamate addition does not have any effect on PHA production in *Omni A* and *Omni B* systems: the PHA biosynthetic pathway seems to be “corrupted” and cannot be relieved by further NADPH supplying.
On the other hand, results obtained with *Omni C* seems to indicate a different scenario in the LS5218 metabolic background: i) the strain is able to produce PHA in MYA; ii) *Omni C* is able to produce PHA also from SB*. Both results indicate that, in this strain, PhaA expression does not interfere with polymer production, although its specific involvement cannot be clearly inferred, since it is reported that LS5218 produces an endogenous thiolase activity. The *fadR* mutation of LS5218 strain, as fact, is responsible of the constitutive expression of *fadA* and *fadB*, which code for the β-ketothiolase activity (Rhie et al., 1995*)*.
Comparison between the amount of PHAs produced by *Lipo C* and *Omni C* in MYA suggests that in *Lipo C* the endogenous 3-ketoacyl-CoA thiolase activity is able to complement the absence of *phaA* allowing PHA production (Rhie et al., 1995*)*. In addition, in *Omni C* PhaA seems to be able to sustain the PHA biosynthetic pathway, helping to produce PHAs: as a fact, PhaA contribute, in *Omni C*, doubling up the PHAs production respect to *Lipo C* (66.8 mg/L Vs 31.2 mg/L). Moreover, ^{1}H NMR analysis of PHA extract from *Lipo C* reveals also that the endogenous 3-ketoacyl-CoA thiolase activity of the LS5218 strain promote the production of only PHB (spectrum not reported). On the contrary, PhaA seems to be able to drive the incorporation of 5-C monomers. To validate this hypothesis GC-MS analysis are requested.

Strain engineering
With the aim to improve polymer production, we have also studied the properties of the principal enzymes involved in monomers fuelling for PHA biosynthetic pathways: PhaA and PhaB.

PhaA
Bacillus cereus genome presents several ORFs putatively coding for β-ketothiolase; one of these genes was the one we used for the construction of the *Omni* systems.

This enzyme presents an identity of 99.7% with another PhaA, which was already characterized (Davis et al., 2008) for being effective in the production of PHAs in a recombinant *E. coli* strain. The substitution of the used PhaA could be an effective strategy to allow the production of PHAs in *Omni A* and *Omni B*. BC5344 was selected as alternative enzyme since, among the β-ketothiolases of *B. cereus,* it revealed the highest identity (46.7 %) with PhaA of *R. eutropha* ($PhaA_{Re}$). $PhaA_{Re}$ is one of the most used PhaA in recombinant system of *E. coli* and it is able to complement PHA biosynthetic pathway with PhaR-PhaC synthase as reported by Tomizawa et al. (Tomizawa et al.,2011). The sequence of BC5344 was amplified from the genome of *B. cereus* 6E/2, but after sequencing it has shown the presence of an internal stop codon. Therefore, another β-ketothiolase of *B. cereus* or other microorganisms, such as *R. eutropha* will be selected and tested.

PhaB

Results obtained with *Lipo* systems had shown a low PHA production with *Lipo C* (Chapter 2.2). The inadequacy of LS5218 strain to produce PHA by the recombinant expression of *sphaRBC* operon from related carbon sources has been also reported by a study (Kihara et al., 2017) in which a recombinant strain of *E. coli* LS5218, containing a biosynthetic operon *RBC* from *B. cereus* (sequence identity to *Lipo*'s operon = 99%) does not produce PHAs. The same systems if it is complemented with a R-hydratase (PhaJ) produces PHAs, suggesting that PhaJ operates as a monomer supplier by modifying products of the β-oxidation cycle (**Figure 1.2.1**) (**Table 3.5.2**).

Since there are not differences between the synthase used by Kihara et al, 2017 and that of *Lipo* system's (PhaR, PhaC), which allow PHAs production in BL21 (DE3) metabolic background, it was hypothesized that different metabolic backgrounds can influence the β-ketoreductase activity (PhaB), in terms of availability of its cofactor (NADPH).

Strain	Synthase	Monomer Supplier	PHA Production	REF
BL21 (DE3)	$PhaR_{B.c.}$-$PhaC_{B.c.}$	$PhaB_{B.c.}$	+	Vastano et al. 2015
LS5218	$PhaR_{B.c.}$-$PhaC_{B.c.}$	$PhaB_{B.c.}$	-	Vastano et al. 2017
LS5218	$PhaR_{B.c.}$-$PhaC_{B.c.}$	$PhaB_{B.c.}$	-	Kihara et al. 2017
LS5218	$PhaR_{B.c.}$-$PhaC_{B.c.}$	$PhaJ_{B.c.}$	+	Kihara et al. 2017

Table 3.5.2: *Scheme of recombinant systems for PHAs production based on B. cereus enzymes.*

Literature about reductase family reports a phylogenetic conservation of two Arginine (RR) in the flavoreductase family which, through the electrostatic interactions with the 2'-phosphate group of the NADPH, control thermodynamics and coenzyme affinity (**Figure 3.5.2-A**). The absence of the two Arg into the cofactor binding site decreases almost three-fold the affinity to NADPH (Sabri et al., 2009).

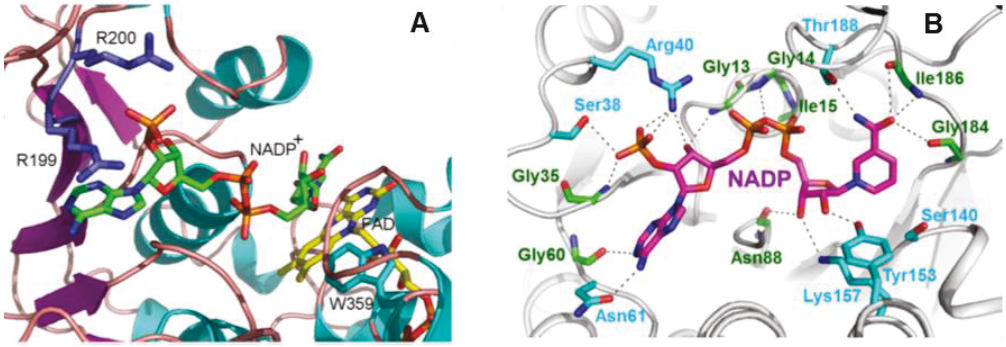

Figure 3.5.2: *A) A magnification of the NADP(H)-binding region of FprA. The side chains of Arg199 and Arg200 make stabilizing electrostatic interactions with the 2'-phosphate group of the NADP+ (Sabri et al., 2009). B) Stabilization of NADP+ (Kim et al., 2014).*

Studies on PhaB from *R. eutropha* ($PhaB_{Re}$) (Matsumoto et al., 2013; Kim et al., 2014) reveal that also this β-ketoreductase present a positive charged residue (Arg) into the cofactor binding site, at the beginning of an alpha-helix domain. This Arg stabilizes the interaction with the 2'-phosphate group of the NADPH (**Figure 3.5.2-B**). An alignment and a comparison between the crystal structure of $PhaB_{Re}$ (Kim et al., 2014) and the 3D model of $PhaB_{Bc}$ structure obtained by Phyre2 tool (Protein Homology/Analogy Recognition Engine V 2.0) show that at beginning of the alpha helix domain in the cofactor binding site of $PhaB_{Bc}$, there are no basic residues turned toward the NADPH binding cavity, which could stabilize the interaction with NADPH; therefore, it was decided to develop a mutant of PhaB (S40R), containing a basic residue into the cofactor binding site.
From the 3D model (**Figure 3.5.3-left**) it was also noticed that there is a basic residue (Lys 41) at beginning of this alpha helix domain, that is pointed outward the cofactor binding site, but that could turn inside if bonded to NADPH, following the same mechanism of a β-ketoreductase from *Synecochoccus elongatus* (**Figure 3.5.4**). Therefore, it was decided to substitute this Lys with an Arg, in order to increase the potential H-bonds involved in interactions with NADPH. These two substitutions lead to an inherent fruitful 2R diade analogous to the one described by Sabri et al. (Sabri et al., 2009). However, since from the 3D model (**Figure 3.5.3-right**) the aminoacids at the beginning of the alpha helix domain, potentially involved in interaction with NADPH, are not clearly identified, two versions of the engineered PhaB were designed (S40R K41R; K41R E42R).

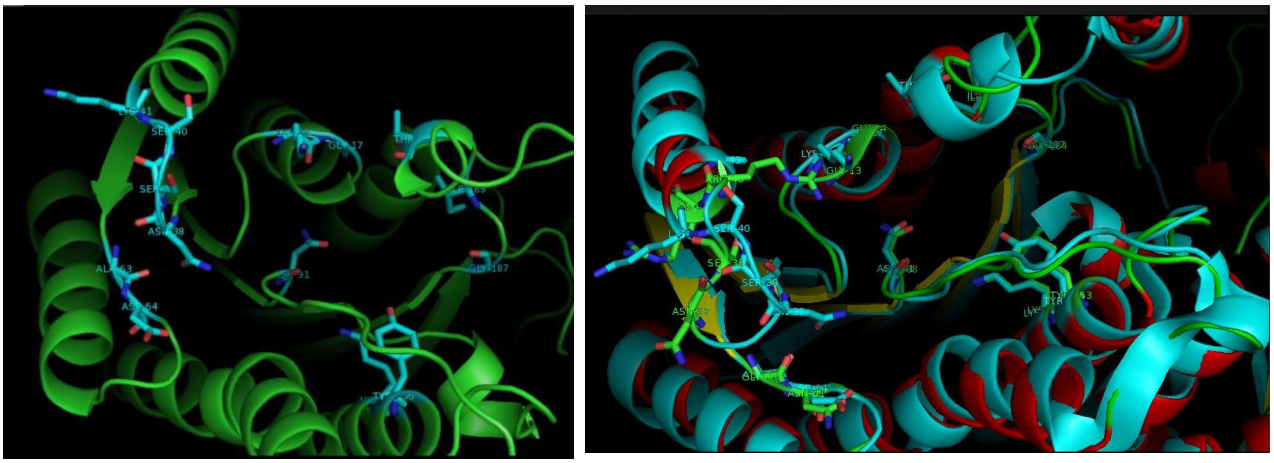

Figure 3.5.3: *Left: Magnification of the NADP(H)-binding region of $PhaB_{B.c}$ realized with Pymol. Aminoacids potentially involved in NADPH binding are sticky coloured. Right: Overlap of the 3D model of $PhaB_{B.c}$ (blue) and Crystal structure of $PhaB_{Re}$ (green).*

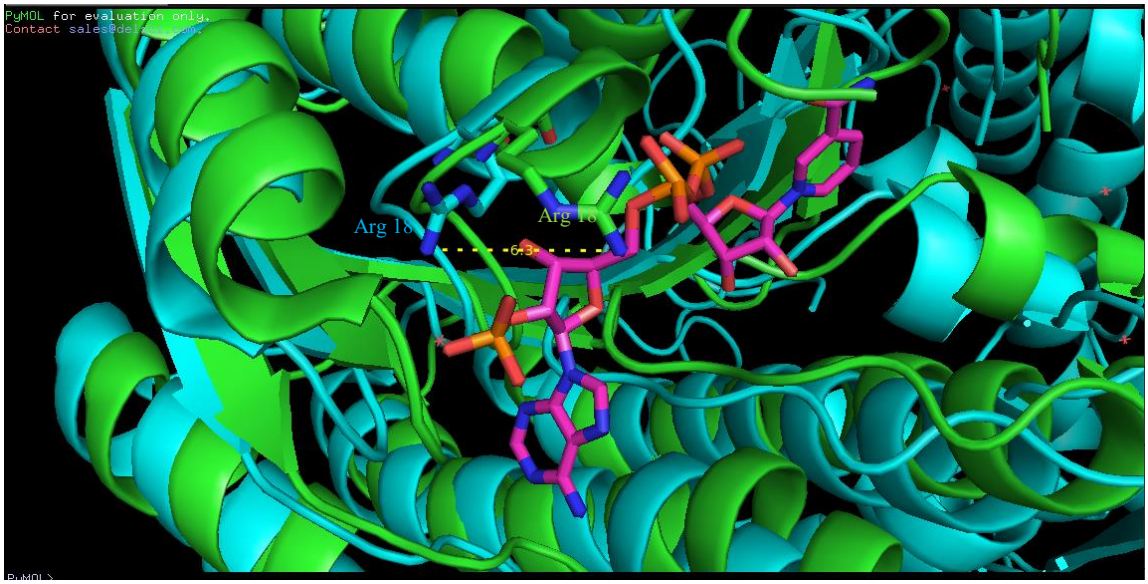

Figure 3.5.4: *Magnification of the NADP(H)-binding region PhaB from S. elongatus. Two versions are overlapped: the structure without the cofactor (Blue) and the structure binding the cofactor (green). The NADPH is reported in sticky. Arg 18 turns inside the cofactor binding site when there is NADPH.*

Conclusions

Glycerol, as main by-product of biodiesel production process, was identified as potential carbon source for PHAs biosynthesis. To this aim *Omni* systems were constructed and their performances compared: production of PHAs was analysed from media boosting both scl and mcl-PHAs production. Moreover, culture media were engineered trough the addition of glutamate to enhance NADPH concentration available for the biosynthetic pathway of PHA production. Although the genes expressed in all the *Omni* systems are the same, *Omni C* produces more PHAs than the others, both from unrelated and related carbon sources: these results suggest that the host strain *E. coli* LS5218 provides a key metabolic background for PHAs production in all the tested conditions.

Considering the data currently available, it is possible to delineate some future perspectives:

- Construction of other *Omni* systems, using different host strains
- PhaA: substitution with different β-ketothiolases, to improve *Omni* systems performances
- PhaB: designed mutants will be constructed and their properties characterized

Once optimized the recombinant systems for production of tailor-made biopolymers, the "*superior cell factories"* will be tested in minimal media containing only glycerol with or without addition of long-chain fatty acids, in order to test them towards the exploitation in industrial conditions.

Materials and Methods

Plasmid, strains and primers	Relevant characteristics/sequence	Source or reference
Plasmids		
pET16SPHA	pET16b derivative, *B. cereus* 6E/2 *sphaRBC*$_{Bc}$	Chapter 2.1
pFLAG-CTS™	*ampr*, P*tac*, pBR322 origin	Sigma-Aldrich
pPTSPHA	pET16SPHA derivate, alternative promoter sequence (*tac*)	Chapter 2.2
pET-40b+phaA	pET40 derivative, *B. cereus* 6E/2 *phaA*$_{Bc}$	Chapter 3.2
pETDuet+sphaRBC+phaA	Dual promoter vector pET16b-pET40 derivative, *B. cereus* 6E/2 *sphaRBC*$_{Bc}$ and *phaA*$_{Bc}$	Chapter 3.2
pPT-40+phaA	pET-40b+phaA, alternative promoter sequence (*tac*)	This study
pPTDuet+sphaRBC+phaA	Dual promoter vector pPTSPHA-pPT40+phaA derivative, *B. cereus* 6E/2 *sphaRBC*$_{Bc}$ and *phaA*$_{Bc}$	This study
Strains		
E. coli Top10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Life Technologies
E. coli BL21(DE3)	F$^-$ *ompT hsdS*$_B$ (r$_B^-$ m$_B^-$) *gal dcm* (DE3)	Novagen
E. coli LS5218	*F*$^+$, *fadR601*, *atoC512*(Const)	Rhie et al. 1995
LipoA	*E. coli* BL21(DE3), pET16SPHA (*sPha*)	Chapter 2.1
LipoB	*E. coli* BL21(DE3), pPTSPHA	This study
LipoC	*E. coli* LS5218, pPTSPHA	This study
OmniA	*E. coli* BL21(DE3), pETDuet+sphaRBC+phaA	Chapter 3.2
OmniB	*E. coli* BL21(DE3), pPTDuet+sphaRBC+phaA	This study
OmniC	*E. coli* LS5218, pPTDuet+sphaRBC+phaA	This study
BL21-A	*E. coli* BL21(DE3), pPT-40+phaA	This study
Primers		
Lac operator Fw_SphI	ATGATTGCATGCGATCCGGGCTTATCGACTGCACGG	This study
Lac operator Rev_NdeI	AATCATCATATGATATCTCCTGTGTGAAATTG	This study
Rev_A9690	CGTAATGCATGCGGTACCTTATAGTAATTCAAACACTCCTGCTG	This study
Fw_BC5344	CGATATCATATGATGAGTAAAACAGTTATTTTAAGTGC	This study
Rev_BC5344	CGATATGCATGCGGATCCTTAGTGAACTTCAATCATCACTGC	This study
PhaBrr Fw40-41	GCGAAAGTAGTTATTAACTATAATAGCCGTCGTGAAGCAGCTGAAAATTTAGTAAATGAATTAGG	This study
PhaBrr_Rev40-41	CCTAATTCATTTACTAAATTTTCAGCTGCTTCACGACGGCTATTATAGTTAATAACTACTTTCGC	This study
PhaBrr Fw41-42	GCGAAAGTAGTTATTAACTATAATAGCAGTCGTCGTGCAGCTGAAAATTTAGTAAATGAATTAGG	This study
PhaBrr_Rev41-42	CCTAATTCATTTACTAAATTTTCAGCTGCACGACGACTGCTATTATAGTTAATAACTACTTTCGC	This study

Tables 3.5.3*: Plasmids, strains and primers*

Cloning

The primers for cloning were obtained from Eurofins Genomics. An MJ Research PTC-200 Thermal Cycler PCR was used for all the PCR carried out. The PCR reactions were set up adapting temperature program to the specific oligos and according to protocol supplied with specific enzyme. PCR were performed by DNA Taq polymerase (Promega) for analytical and by DNA Phusion High-

Fidelity Polymerase (NEB) for plasmid construction. The plasmidic DNA preparations were performed using the QIAprep Spin Miniprep Kit (QIAGEN).

pPT-40+phaA vector and BL21-A
The sequence containing the *tac* promoter was amplified by PCR using *pFLAG-CTS* as template. Forward and Reverse primers added respectively the restriction sites for *Sph*I and *Nde*I. Ligation of digested amplicon and vector pET-40+phaA was set up to obtain the *pPT-40+phaA* vector. The ligation product was transformed in TOP 10 *E. coli* strain and screened by colony PCR and by a double. The so obtained recombinant vector was transformed in BL21 (DE3) *E. coli* strain obtaining *BL21-A* strain. PhaA over-Expression in *BL21-A* was verified by SDS-PAGE.

pPTDuet+sphaRBC+phaA vector
The *pPT-16+sphaRBC* vector presents a unique restriction site for *Sph*I upstream of the *sphaRBC* operon. The sequence containing the *tac* promoter and *phaA* coding gene was amplified by PCR from the *pPT-40+phaA* vector. The Reverse primer added a restriction site for *Sph*I. The amplified sequence and the vector were digested by *Sph*I. After the vector dephosphorylation by CIP (NEB), ligase reaction was set up to obtain the pPTDuet+sphaRBC+phaA vector.The vector was transformed in Top 10 *E. coli* strain and colonies were screened on an agarose gel following the protocol described by Koul et al. (Koul et al., 1997). In recombinant clones, the correct orientation of the insert was verified by a double digestion with *Kpn*I and *Hpa*I.

BC5344 amplification
Genomic Extraction from Bacillus cereus E6/N was performed as describe in Chapter 2.1. The amplified sequence containing the *BC5344* gene was sequenced by “Eurofins Genomics”, but it contained a stop codon, encoding for a for an inactive protein.

Design of PhaB mutants
Two mutants of PhaB were designed to introduce, through the “quick-change” system (QuikChange II Site-Directed Mutagenesis Kit-Agilent), the double Arg: the mutagenic oligos were projected to take the substitution of aminoacids 40 and 41 in one, and the substitution of aminoacids 41 and 42 in the other.

Media
LB Composition for 1 L: 5g of Yeast Extract; 10g of Bacto Tryptone and 10 g of NaCl. **MM** Composition for 1 L: 0.5 g of Yeast Extract; 1 g of Bacto Tryptone and 1 g of NaCl. Saline Buffer* (**SB***) Composition for 1 L: 100 mL M9 salt [10 x]; 0.1 mL of $CaCl_2$ [1 M]; 2 mL of $MgSO_4$ [1 M], 2 mL of $FeSO_4$ [5 mM]; 20 g Glycerol; 2 g of Yeast Extract. Sterilized solution of mixed long-chain fatty acids (SIGMA) was added to a final concentration of 0.1 % (w/v). **Composition** of M9 salt [10x] for 1 L [pH 7.4]: 60 g Na_2HPO_4; 30 g KH_2PO_4; 5 g NaCl; 10 g NH_4Cl. **MYA** Composition for 1 L: 6 g of Na_2HPO_4; 3 g of KH_2PO_4; 1.4 g of $(NH_4)_2SO_4$; 0.5 g of NaCl; 0.2 g of MgSO4*7H_2O; 10 g of Yeast Extract; 5 g of casein amino acids; 30 g of Glycerol.

PHA Production
Recombinant cells from solid culture were inoculated in 20 mL LB medium in shaken flask of 100 mL. This preculture was grown for one night at 37° C on a rotary shaker (200 rpm), then a volume of suspension sufficient to reach a final Optical Density (OD_{600}) value of 0.1 was used to inoculate shaken flasks of 250 mL containing 50 mL of different culture medium. The induction of protein expression has been carried out with 0.5mM of IPTG after 5 h in SB* and 3 h in MYA (except for OmniC system, which needs 2 mM of IPTG in MYA medium with addition of glutamate). Cells, cultured at 37°C on a rotary shaker (220 rpm), have been collected after 72h from induction and lyophilized for PHAs extraction. To determine PHA’s content of the fermented dry cells in each flask, the biopolymer was extracted by vigorously shaking dry cell pellets (100 mg), mixed with chloroform (30 mL) in a shaking flask overnight at room temperature, with constant stirring. After removing the cell debris by filtration through Whatman no.1 filter paper, the clear chloroform extract was dried. The polymer was then re-dissolved in 5 mL of chloroform and precipitated using 10 volumes of methanol as a precipitating solvent.
NMR
^{1}H NMR spectra were recorded on Bruker DRX-400 (^{1}H NMR: 400 MHz) in CDCl3 (internal standard, for ^{1}H: CHCl3 at d 7.26 ppm).

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4. Concluding remarks

The PhD project, developed and described in this thesis, was focused on multiple aspects of biopolymers life. As fact efforts were devoted on biopolymers sustainable synthesis, functionalization and degradation. The achieved results can be summarized as reported below:

- Recombinant PHAs producing strains were designed and constructed to shed light on the influence of i) the promoter driving bio-synthetic operon and ii) the "host metabolic background" in biopolymers production.

- Enzymatic approach for biopolymer functionalization was applied. The functionalised DMI-PHA, PEG-PHA and PEG-DMI-PHA polymers obtained and characterised in this study open new perspectives for the use of PHAs as biodegradable and biocompatible materials of choice for biomedical applications.

- *T. cellulosilytica* cutinase 1 (Thc_Cut1) was successfully immobilized via His-tag on three different Fe(III)-activated carriers and applied for oligoesters synthesis. Moreover, the recyclability of the preparation was verified with a solventless approach.

- The Thc_Cut1 wild type and its mutants, recombinantly expressed in *P. pastoris*, were studied for aromatic and aliphatic polyesters degradation. Interesting results came out revealing improved hydrolytic performances for designed variants.

- Biorefinery target was chased by multiple engineering strategies to obtain systems suitable for PHA production from waste-derived carbon sources: i) exhausted oils and ii) crude glycerol.

- An innovative sustainable Bioprocess for valorisation of waste oils with high content of free fatty acids was exploited on both recombinant and native PHAs producers. Obtained results pay the way for the establishment of a new business idea (start-up).

5.Appendix

Communications

- M. Vastano, F. Sannino, E. Parrilli, M. L. Tutino, C. Pezzella and G. Sannia, " **Polyhydroxyalcanoates from psychrophylic bacteria**" Italian Forum on Industrial Biotechnology and Bioeconomy - IFIB Naples, 22 - 23 October 2013. **Poster**
- M. Vastano, A. Casillo, M. M. Corsaro, G. Sannia and C. Pezzella, "**New systems for polyhydroxyalkanoates production from related carbon sources: valorisation of waste materials** " 8th International Congress on Polymer and Fiber Biotechnology - ICPFB Braga, Portugal, 25-27 May 2014. **Poster**
- M. Vastano, A. Casillo, M. M. Corsaro, G. Sannia and C. Pezzella, "**New clues to design cell factories for tailor-made biopolymer production: *Bacillus cereus* as a source of polyhydroxyalkanoates biosynthetic proteins"** 16th European Congress on Biotechnology in Edinburgh, 13-16 July 2014. **Poster**
- M. Vastano, A. Casillo, M. M. Corsaro, G. Sannia and C. Pezzella, "**Production of medium-chain-length polyhydroxyalkanoates from waste oils by recombinant *Escherichia coli* "** OliPHA: Functional Sustainable Packaging; Workshop and Demo, Pisa, 12-13 May 2015. **Poster**
- M. Vastano, A. Casillo, M. M. Corsaro, G. Sannia and C. Pezzella, "**Production of medium-chain-length polyhydroxyalkanoates from waste oils by recombinant *Escherichia coli* "** IFIB 2015, Lodi, 24-25 September 2015. **Poster**
- M. Vastano, I. Corrado, A. Pellis, G.M. Guebitz, G. Sannia and C. Pezzella, **"Circular Economy: Polyhydroxyalkanoates synthesis for a sustainable biodiesel production process "**, Workshop "THIRD GENERATION BIOREFINERIES", Fondazione Golinelli (Bologna) 23- 24 March 2017. **Oral**
- M. Vastano, A. Pellis, B. Immirzi, G. Dal Poggetto, M. Malinconico, G.M. Guebitz, G. Sannia, and C. Pezzella, **"Recombinant production and derivatization of amorphous mcl-Polyhydroxyalkanoates: innovative enzymatical approach for polymer bio-functionalization "**, Designer Biology from proteins and cells to scaffolds & materials, Vienna, Austria, 7-9 June 2017. **Oral**
- M. Vastano, I. Corrado, C. Pezzella and G. Sannia, **"NoW BioFP: No Wastes Bioprocess for conversion of low value substrates into Biofuels and Biopolymers"**, StartUp Initiative Intesa Sanpaolo BioCircular Economy Arena Meeting, Milano, Italia, 22 November 2017. **Oral**

Publications

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Other activities

- "Bio-UNIVERSE". **Scientific conference organization**
- " XXIX Futuro Remoto; Un viaggio tra scienza e fantascienza, una festa di Arte Scienza Tecnologia. Napoli 15-19 October 2015. **Scientific dissemination**
- " XXX Futuro Remoto; Un viaggio tra scienza e fantascienza, una festa di Arte Scienza Tecnologia. Napoli 7-10 October 2016. **Scientific dissemination**
- "CAMPANIA NEWSTEEL - DALLA RICERCA ALL'INNOVAZIONE"; Call for business idea proposed by PhD students. **Business incubation process at Campania NewStell**
- Città della scienza; presentation of Seewty Biorefinery, board game based on BEETOUT project financed by Fondazione CARIPLO. Napoli 21 October 2017. **Scientific dissemination**

Stage in foreign laboratories

- **Visiting PhD student** at "University of Natural Resources and Life Sciences, Vienna- Department of Agrobiotechnology, IFA-Tulln. *November 2016-March 2017*

Alternative solvent for sustainable PHA extraction: γ-valerolactone

This PhD work was also focused on testing alternative, low-environmental impact downstream methods, avoiding the consumption of hazardous chemical, and favoring the choice of solvents, potentially derived from renewable biomasses (Horvàth et al., 2008). In this frame, interesting results were achieved with γ-valerolactone. This compound, obtainable from levulinic acid, is a naturally occurring chemical in fruits and a frequently used food additive, and it has been already applied as PHB solvent for heritage application (Samorì et al., 2016). At best of our knowledge no attempts to use γ-valerolactone for direct extraction of PHAs from cells have been reported. In preliminary trials γ-valerolactone was applied in standard PHA extraction protocol instead of chloroform. Two different lyophilized biomasses were extracted: i) *E. coli* containing PHB-co-PHHX and ii) *P. resinovorans* containing mcl-PHAs. 30 and 100 mg of cells were extracted with 30 mL of γ-valerolactone at 30 °C for 16 h. Control extraction were performed with 30 mL of chloroform in the same conditions. All the extracts were treated with cold methanol to induce PHAs precipitation. PHAs were recovered from all samples treated with γ-valerolactone and it is worth of note that a low volume of methanol was adequate to completely precipitate the extracted biopolymers. As fact, while it is extensively reported a chloroform: methanol ratio of 1:10 for PHA precipitation, the obtained results claimed that a 1:5 ratio is enough for biopolymers recovery.

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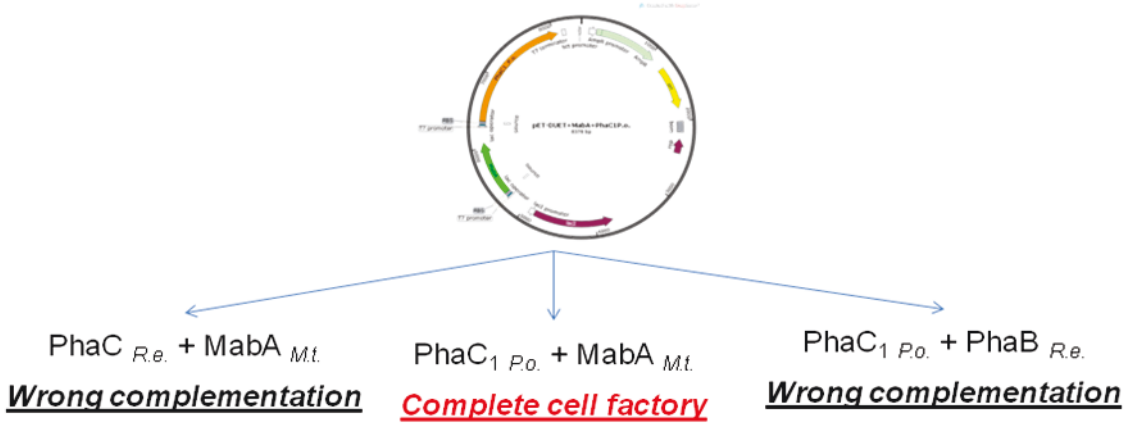
Isolation of new PHAs biosynthetic enzymes by using an innovative selection method based on a metagenomics approach

A side project of this PhD thesis was the selection of mcl-selective PHA synthetic genes from metagenomic libraries, by means of high-throughput screening method. The power of this screening approach relies on the designing of properly engineered *E. coli* strains ("scaffold strains") to be used as host for the functional screening of mcl-selective PHA synthetic genes from metagenomic libraries.
In PHAs producing cells, the metabolic steps determining selection of specific monomers (scl or mcl) are:
i) routes fuelling precursors for mcl-monomers
ii) the polymerization step of mcl-monomers into PHAs
An "incomplete cell factory" can be created if only one of the two PHA synthetic genes required for each of the above-mentioned steps is provided to the host.



	mcl	scl
Monomers fuelling	MabA $_{M.t.}$	PhaB $_{R.e.}$
Polymerization	PhaC1 $_{P.o.}$	PhaC $_{R.e.}$

Figure 1: *Genes selected and systems constructed for set-up of innovative selection method based on metagenomic approach*

In order to construct the scaffold strains, a genome mining approach was used to select the genes coding for proteins with mcl-specificity. A "complete cell factory" (**Ccf**) expressing both mcl-selective proteins selected: MabA$_{M.t.}$ and PhaC1$_{P.o.}$ was constructed. While PhaC1$_{P.o.}$ from *Pseudomonas oleovorans* has been already characterized for mcl-PHAs production (Huisman et al., 1991); MabA$_{M.t.}$ is a β-ketoreductase from *Mycobacterium tuberculosis* specific for medium-long chain substrates (Labesse et al., 2002) which had been never implemented in PHAs biosynthetic pathway as monomers fuelling enzyme. Two other systems, named Wrong complementation (**Wc'** and **Wc"**) were constructed as negative control of the screening conditions optimization. Enzymes from *Ralstonia eutropha* (PhaC$_{R.e.}$ and PhaB$_{R.e.}$), already characterized for scl-selectivity, were selected for **Wc** strains

construction. PHAs biosynthetic pathway in **Wc'** and **Wc"** are respectively composed by the dyads PhaC*R.e*-MabA$_{M.t.}$ and PhaC1$_{P.o}$-PhaB*R.e.*(**Figure 1**). These systems mime the clone for which to scaffold strains would be transformed with metagenomic genes endowed with the wrong monomer selectivity.
All these systems were tested by a fluorescent based assay, fatty acids salts (8, 10, 12, 16 and 18 carbon atoms long) were exploited to this aim but no one gave a positive answer.
The producing capability of *Ccf* system was then investigated in liquid medium. Screening of several induction conditions revealed the inability of the *Ccf* system to co-express the two biosynthetic proteins. In order to obtain a system able to produce both proteins simultaneously, the genes transcription was split into 2 different compatible vectors both under the control of a *T7* promoting sequence. A co-transformed recombinant strain was obtained and its characterization is under investigation.

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