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production bactérienne à façon et biodégradabilité :  
étude des communautés bactériennes et des mécanismes  
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Gabrielle Derippe

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# THESE DE DOCTORAT DE

**UNIVERSITE BRETAGNE SUD**

**ECOLE DOCTORALE N° 647**

*Sciences pour l'Ingénieur*

Spécialité : *Génie des Procédés et Bioprocédés*

Par

**Gabrielle Derippe**

**Les polyhydroxyalcanoates (PHA) en milieu marin :  
production bactérienne à façon et biodégradabilité**

Etude des communautés bactériennes et des mécanismes de  
biodégradation impliqués

**Thèse présentée et soutenue à Lorient, le 5 octobre 2023**

**Unité de recherche : Institut de Recherche Dupuy de Lôme (IRDLD) – UMR CNRS 6027**

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# Publications

## Publiée ou Soumise

Odobel C, Dussud C, Philip L, **Derippe G**, Lauters M, Eyheraguibel B, Burgaud G, Ter Halle A, Meistertzheim A-L, Bruzaud S, Barbe V, Ghiglione J-F. **Bacterial Abundance, Diversity and Activity During Long-Term Colonization of Non-biodegradable and Biodegradable Plastics in Seawater**. *Frontiers in Microbiology* **2021**, 12, 734782 (IF 5,640)

*Publiée*

**Gabrielle Derippe**, Léna Philip, Pierre Lemechko, Anne-Leïla Meistertzheim, Valérie Barbe, Stéphane Bruzaud and Jean-François Ghiglione. **Marine biodegradation of various tailor-made polyhydroxyalkanoates (PHA) is influenced by the chemical structure and associated bacterial communities**

*Soumise le 28/06/2023 à Journal of Hazardous Materials (IF 14,220) - En correction*

## En préparation

**Gabrielle Derippe**, Boris Eyheraguibel, Jean-louis Petit, Véronique De Berardinis, Valérie Barbe, Stéphane Bruzaud and Jean-François Ghiglione. **Enzymatic degradability of two short-chain length polyhydroxyalkanoates (PHA) by a novel depolymerase isolated from the marine bacteria *Alteromonas plasticoclasticus***.

*Soumission prévue le 30/09/2023 à Applied Microbiology and Biotechnology (IF 5,560)*

**Gabrielle Derippe**, Timothée Le Men, Pierre Lemechko, Jean-François Ghiglione, Valérie Barbe and Stéphane Bruzaud. **A marine bacteria able to synthesize both short- and medium-chain length polyhydroxyalkanoates**.

*Soumission prévue le fin 2023 à International Journal of Biological Macromolecules (IF 8,025)*

Léna Philip, Karine Lebaron, Isabelle Calves, Edouard Lavergne, **Gabrielle Derippe**, Pierre Lemechko, Stéphane Bruzaud, Mireille Pujo-Pay, Pascal Conan, Mounir Traïka, Boris Eyheraguibel, Valérie Barbe, Anne-Leïla Meistertzheim & Jean-François Ghiglione. **Influence of PHA composition on their biodegradability under marine conditions**.

*Soumission prévue le 15/09/2023 à Marin Pollution Bulletin (IF 7,001)*

Valérie Barbe, Justine Jacquin, Adèle Wolinski, **Gabrielle Derippe**, Jinguang Cheng, Corinne Cruaud, David Roche, Madeleine Bouzon-Bloch, Jean-Louis Petit, Pascal Conan, Mireille

Pujo-Pay, Stéphane Bruzaud, Jean-François Ghiglione. Bioplastic degradation and assimilation processes by a novel bacteria isolated from the marine plastisphere.

*Soumission prévue le 01/09/2023 à Chemical engineering journal (IF : 16.744)*

# Communications orales et poster

## Présentations orales en conférence

### **1. International Symposium on Biopolymers (ISBP) - 01.07.2021**

**Derippe G et al.**, PHA biodegradation in marine environment: an innovative approach (webinaire)

### **2. Journées de la section Grand Ouest du Groupe Français d'Etudes et d'Applications des Polymères (GFP) – 08.07.2021**

**Derippe G et al.**, De la biodégradation en milieu marin des polyhydroxyalcanoates vers une production « à façon » (webinaire)

### **3. 10<sup>ème</sup> colloque de l'Association Française d'Ecologie Microbienne – 19.11.2021**

**Derippe G et al.**, Biodégradabilité en milieu marin de différents plastiques biosourcés produits « à façon » (webinaire)

### **4. Groupement de Recherche Polymères et Océans, Brest – 29.06.2022**

**Derippe G et al.**, Biodégradabilité de PHA « sur mesure » en milieu marin

### **5. MICRO 2022 – 15.11.2022**

**Derippe G et al.**, « Tailor made » polyhydroxyalkanoate production and their associated biodegradation in marine environment

### **6. 50<sup>ème</sup> colloque du Groupe Français d'Etudes et d'Applications des Polymères (GFP) – 23.11.2022**

**Derippe G et al.**, Biodégradation de scl et mcl PHA en milieu marin contrôlé

### **7. 7 International symposium on Environmental Biotechnology and Engineering – 23.05.2023**

**Derippe G et al.**, Production of custom short chain and medium chain-length polyhydroxyalkanoates and their associated biodegradation in natural marine environment

## Poster en conférence

### **8. International Symposium on Biopolymers (ISBP) - 14.09.2022**

**Derippe G et al.**, Biodegradation of « tailor-made » polyhydroxyalkanoates in marine environment

# Encadrements et supervisions

## 1. **Timothée Le Men** – Université Bretagne Sud

Master 1 Biotechnologie

Production de scl- et mcl-PHA par une nouvelle souche bactérienne : *Alteromonas plasticoclasticus MED1*

Mars 2022 à Mai 2022

## 2. **Romane Fravallo** – Université Bretagne Sud

Master 1 Biotechnologie

Optimisation de la production de scl- et mcl-PHA par *Alteromonas plasticoclasticus MED1*

Mars 2023 à Mai 2023

## 3. **IMBRSea summer school** – Master 2 Internationaux IMBRSea – Tjärnö, Suède

Encadrement projet de recherche en biologie marine d'un groupe de 6 étudiantes Master 2 – Du 1<sup>er</sup> au 12 Aout 2022.

Projet visant à évaluer la pollution en micro- et macro-plastiques sur des plages de la baie de Tjärnö différemment impactées par des activités anthropogéniques.

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# Préface

La production de matières plastiques a dépassé les 390 millions de tonnes par an à partir de 2021 et ne cesse d'augmenter. Ces matériaux aux propriétés versatiles et aux coûts de fabrication faibles se retrouvent dans tous les secteurs d'activités. Que ce soit dans les secteurs de l'emballage, du médical ou des transports, le progrès apporté par ces matières synthétiques est indéniable. Paradoxalement aux bénéfices apportés par l'introduction massive des matériaux plastiques à notre société, leur fin de vie représente un inconvénient majeur d'un point de vue environnemental. Les plastiques dits conventionnels, c'est-à-dire issus de ressources pétrochimiques sont pour la plupart non-biodégradables dans des temps raisonnables. Après usage, ils sont le plus souvent incinérés (ce qui génère des fumées toxiques et polluantes), enfouis dans des décharges ou bien terminent leur vie dans l'environnement. Lorsque leur collecte est mal gérée ('mismanaged plastic waste'), le milieu marin est le réservoir final de ces déchets à longue durée de vie où une quantité importante (environ 10% de la production mondiale) y est accumulé, dont 80% proviennent des continents. Que ce soit la persistance liée à la structure chimique des matières plastiques ou encore la toxicité des additifs que les plastiques contiennent, l'impact négatif qu'ils génèrent sur l'environnement marin est clairement avéré, soutenu par une bibliographie abondante dans ce domaine. Etouffement ou strangulation par les macroplastiques ou ingestion des microplastiques et des nanoplastiques, la faune marine est particulièrement touchée par cette pollution.

Des solutions de lutte contre la pollution plastique ont récemment été mises en place au niveau gouvernemental et sociétal dans de nombreux pays afin de limiter l'impact des plastiques sur l'environnement. Parmi ces solutions, la réduction des plastiques à usage unique a fait l'objet de plusieurs lois. Un exemple est l'interdiction des sacs en plastique jetables dès 2015. Plus récemment, en janvier 2021, l'interdiction des pailles en plastique, de vaisselles jetables ou cotons de tiges est mise en vigueur en France et plus globalement dans l'union européenne. Dernièrement, la loi AGEC, appliquée en 2023, vise encore plus à limiter la production de plastique à usage unique à travers la réduction, le réemploi et le recyclage des déchets. Le recyclage des matières plastiques constitue également une réponse à la pollution plastique. Néanmoins, la collecte mais aussi les limites des matières plastiques à être retransformées et réutilisées possède quelques contraintes techniques et économiques. Enfin, une prise de conscience sur notre consommation se met progressivement en place à l'échelle

individuelle et collective et tend à diminuer l'utilisation de plastique à usage unique en lien avec le secteur de l'emballage.

D'autres solutions, telle que le développement de plastiques biodégradables, se profilent depuis les dernières décennies pour répondre à des secteurs d'activités ne pouvant se passer de plastiques. Parmi les plastiques biodégradables, les polyhydroxyalcanoates (PHA) sont des polymères d'origine bactérienne et biosourcés qui ont montré de bonnes capacités de biodégradation dans divers types d'environnements. Ils pourraient constituer une des réponses à la pollution plastique dans des usages bien définis et adéquates tels que les cosmétiques, les accessoires de pêche (filets et bouées entre autre) ou encore le paillage en agriculture dont une quantité considérable de ces matériaux finissent leur vie dans l'environnement.

Bien que les taux de biodégradation des PHA ait été déjà étudiés dans le sol ou en conditions de compostage, les études dans l'environnement marin sur la biodégradation de ces polymères sont limitées et se concentrent exclusivement sur des tests de respirométrie (production de CO<sub>2</sub>) ou des transformations physiques (perte de masse). De plus, l'étude du biofilm bactérien qui colonise ces plastiques et qui sont responsables de leur biodégradation est quasi-inexistante. Enfin, les taux de biodégradation déterminés ne concernent qu'un certain type de PHA (les PHA à chaînes courtes, *scl*-PHA), du fait du manque de disponibilité de l'autre type de PHA (les PHA à moyennes chaînes, *mcl*-PHA) qui ne sont pas commercialisés et nécessitent des productions en laboratoire.

Afin de mieux définir l'usage de ces polymères et de mieux comprendre les interactions que peuvent générer leur éventuelle fin de vie dans l'environnement, les mécanismes de leur biodégradation ainsi que l'étude des microorganismes associés méritent d'être mis en lumière. Les connaissances apportées sur le comportement de ces polymères dans l'environnement marin en fonction des propriétés intrinsèques qu'ils peuvent présenter sont nécessaires pour adapter leur production et mieux définir leurs usages.

Pour répondre à ces questions, l'Institut de Recherche Dupuy de Lôme (IRDL) et le Laboratoire d'Océanographie Microbienne (LOMIC) ont conjointement développé le projet PHABIOMAR. L'objectif visé dans cette collaboration était d'aborder l'étude de la biodégradation des PHA dans l'environnement marin à l'aide de compétences multidisciplinaires et complémentaires : la science des polymères et la microbiologie. Grâce à un financement du CNRS obtenu dans le cadre de l'édition 2020 du programme MITI 80|PRIME, ce projet réalisé au travers de cette thèse vise à contribuer au développement de

PHA présentant des propriétés physico-chimiques distinctes (et donc aux usages multiples) et d'évaluer leur degré de biodégradabilité en milieu marin. Le projet aborde la biodégradation des PHA en milieu marin de façon holistique en commençant par la production de PHA « à façon » en culture pure à partir de différentes souches bactériennes, sources de carbone et procédés. Le cœur du projet réside ensuite dans l'étude de leur biodégradation en milieu naturel afin de mieux identifier et comprendre le rôle des communautés bactériennes associées. Enfin, à l'échelle macromoléculaire, certains mécanismes de dépolymérisation *in vitro* ont été approfondis grâce à la production et la caractérisation d'enzymes impliquées dans la dégradation des PHA. Au cours de ce travail de thèse, une attention particulière s'est portée sur une nouvelle souche bactérienne *Alteromonas plasticoclasticus MED1* qui s'est avérée présenter des capacités originales de production et de dégradation des *scl*-PHA et *mcl*-PHA.

Ce travail de thèse est présenté en 5 chapitres qui relatent des approches complémentaires utilisées dans cette thèse, faisant appel aux disciplines scientifiques de la chimie des polymères, de l'écologie et écotoxicologie microbienne marine, de la génétique et de la biologie moléculaire.

Le premier chapitre dresse un état de l'art des connaissances sur les principales matières plastiques et plus particulièrement sur les PHA. Une attention particulière a été portée sur les voies métaboliques impliquées dans la production et la dégradation des PHA.

Les chapitres suivants traitent des données acquises à partir des expérimentations réalisées lors de cette thèse. Ils sont décrits sous forme d'articles scientifiques, après une brève introduction de la problématique de chaque chapitre.

Le chapitre 2 relate de la production de différents *scl*- et *mcl*-PHA à partir de deux souches bactériennes utilisant plusieurs sources de carbone. Il aborde également leur biodégradation en conditions naturelles dans l'environnement marin. Différents tests de biodégradabilité y sont décrits pour chacun des PHA, ainsi que le suivi des communautés bactériennes associées. La corrélation entre le degré de biodégradabilité et la famille des PHA concernée (*scl*- pour *short chain-length* et *mcl*- pour *medium chain-length*) est décrite.

Le chapitre 3 aborde la biodégradation des *scl*-PHA d'un point de vue macromoléculaire. La production d'enzymes spécialisées (les PHA dépolymérase) à partir de trois souches bactériennes est décrite. Une nouvelle enzyme appartenant à la bactérie marine *Alteromonas plasticoclasticus MED1* est caractérisée et ses caractéristiques sont comparées à

deux autres enzymes précédemment étudiées dans la littérature. L'influence de la cristallinité des *scl*-PHA sur la cinétique de dégradation des enzymes a été évaluée.

Le chapitre 4 focalise sur la capacité de production de PHA chez *Alteromonas plasticoclasticus* *MEDI*, concomitantes à ses capacités de dégradation. A partir de différents substrats, nous montrons que cette bactérie possède la capacité originale de produire des *scl*-PHA ainsi que du *mcl*-PHA. Les voies métaboliques impliquées dans ces productions y sont présentées.

Les conclusions et perspectives de ce travail de thèse sont exposées dans le chapitre 5.

# Chapitre 1 : Contexte de l'étude

## 1 Les plastiques et leur persistance dans l'environnement

### 1.1 Avènement des matières plastiques

C'est en 1833 que le Français Henri Braconnot inventa le nitrate de cellulose qui servira dès les années 1860 à remplacer l'ivoire dans les boules de billard (Chalmin, 2019). Le premier plastique synthétique, la bakélite, apparaîtra au début des années 1900 reprenant le nom de son inventeur, le chimiste américain Léo Hendrik Baekeland (Baekeland, 1909). De nombreux plastiques aux propriétés variées furent découverts dans les décennies suivantes : le poly(chlorure de vinyle) (PVC) en 1913 encore très utilisé dans le bâtiment, le polyéthylène (PE) en 1933, le polyamide 6,6 (ou Nylon 6,6) en 1935 qui remplacera la soie et la laine de nombreux vêtements après avoir grandement servi dans la conception de toiles de parachute durant la deuxième guerre mondiale. S'en suivent la mousse polystyrène (PS) ou encore le poly(éthylène téréphtalate) (PET) en 1941 qui constitue la majorité des bouteilles en plastique produites et encore en production et le polypropylène (PP) en 1957, omniprésent dans le secteur de l'emballage (Baker, 2018).

Durant les trente glorieuses la production de matières plastiques connaît un véritable essor conséquemment à des pénuries d'autres matériaux à la suite de la seconde guerre mondiale. La production de masse de plastique commença dès les années 1950 avec une croissance annuelle de 15% jusqu'en 1970 puis d'une croissance moyenne de 8.3% de 1950 à 2017 (Geyer, 2020). Elle était comprise entre 4 et 8 millions de tonnes (Mt) avant 1950. Elle dépasse les 390 Mt en 2021 (Plastic Europe, 2023).

Cette production croissante est dû à la versatilité que peuvent arborer tous les types de plastiques, leur légèreté, leur résistance ainsi que leur faible coût de production. Les matières plastiques se retrouvent dans tous les domaines de la vie et certains chercheurs associent même leur industrialisation au début de l'ère géologique de l'anthropocène (Zalasiewicz et al., 2016). Les industries du bâtiment sont de gros consommateurs de plastiques et représentent 20% de la production totale, que ce soit à base de PVC pour les tuyaux ou encore de polyuréthane pour la mousse isolante des habitations (Plastic Europe, 2021). Dans les transports, le plastique trouve également une solide place en tant que matériaux léger et performant, que ce soit dans les



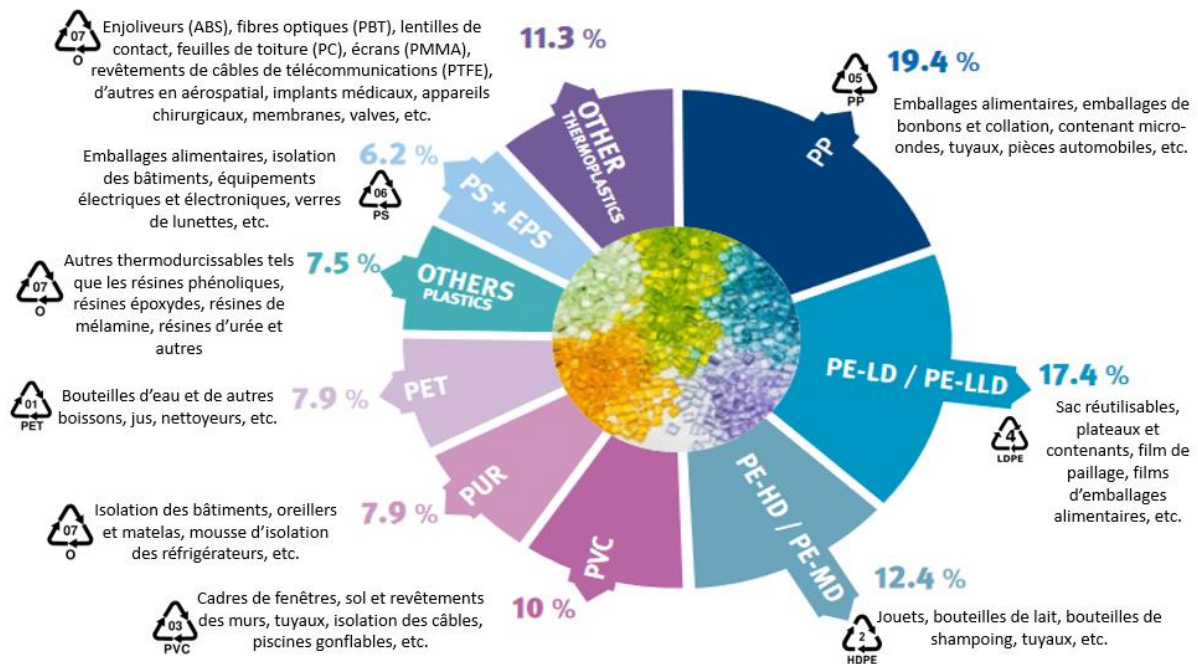
voitures, les trains, les bateaux ou encore les avions. La demande de plastique en 2019 dans l'automobile est estimée à presque 10% en Europe (Plastic Europe, 2021). Les fibres synthétiques, principalement composées de PET, sont aujourd'hui le matériau de choix au détriment des fibres naturelles telles que le coton, la laine ou le lin pour l'industrie du textile qui produit chaque année plus de 70 Mt de plastique (Beckman, 2018). D'autres champs d'applications font appel aux matières plastiques tels que l'agriculture, l'électronique ou le médical apportant un véritable progrès que ce soit d'un point de vue économique ou encore sanitaire. Le principal consommateur de plastique reste cependant le secteur de l'emballage qui représentait en 2019 presque 40% de la demande en consommation de plastique (Plastic Europe, 2021). Bien que cette consommation de matières plastiques tend à stagner en Europe suite à diverses réglementations politiques, les pays occidentaux restent les principaux consommateurs et la production mondiale continue d'augmenter chaque année (Plastic Europe, 2023).

## 1.2 Le plastique

De nombreuses définitions existent pour le terme « plastique ». Un consensus se retrouve dans le fait qu'il s'agit de larges molécules synthétiques résultant de la polymérisation de monomères composés de milliers d'atomes de carbones combinés à des atomes tels que l'hydrogène, l'azote, l'oxygène, le soufre, le chlore ou le fluor bien souvent issus de la pétrochimie (Alauddin et al., 1995). Leur particularité est également d'être formables et/ou déformables sous l'effet de la température. Deux catégories principales de plastiques sont distinguables ; les plastiques dits thermodurcissables et les plastiques dits thermoplastiques (Alauddin et al., 1995). La première catégorie renferme les plastiques qui deviennent solides une fois fondus sous l'effet de la température. Ce changement d'état est irréversible. Une fois le polymère solidifié, les molécules sont chimiquement liées et forment un gigantesque réseau de molécules. C'est le cas de nombreux polyesters, silicones ou polyuréthanes (Alauddin et al., 1995). *A contrario* les thermoplastiques peuvent être remodelés en fonction de la température. Les molécules ne sont pas liées chimiquement et chaque chaîne de polymère est indépendante. Sous l'effet de l'augmentation de la température, les thermoplastiques tendent à être plus modelables et inversement, plus durs sous l'effet d'une basse température. Bien que l'action de durcissement des thermoplastiques ne soit pas irréversible, le nombre de cycles alternant des changements de températures n'est pas infini et peut, *in fine*, altérer les propriétés même du plastique et/ou son esthétique (Alauddin et al., 1995). Les principaux thermoplastiques sont le PE, le PS ou le PP.

## a. Les plastiques non biodégradables

Les plastiques non biodégradables représentent la majorité des plastiques utilisés. Il en existe 5 principaux : le PE, le PP, le PS, le PET et le PVC et constituent à eux cinq 67.2% de la demande en plastique en Europe en 2019 (Fig. 1) (Plastic Europe, 2021).



**Figure 1 :** Pourcentage de la demande des différents types de plastiques en Europe en 2019 et leurs principales applications. Adaptée de Plastic Europe, 2021

Le PE toute formes confondues représente 29.8% de la demande en plastiques en Europe en 2019 (Fig. 1). Les procédés de fabrication du PE permettent d'obtenir différentes densités et donc différentes applications allant de la fabrication de jouets solides, des bouteilles de shampoing ou autres contenant de la sorte ou encore des films alimentaires ou de paillages dans le domaine de l'agriculture (Fig. 1) (Plastic Europe, 2021). Sa fabrication résulte de la polymérisation d'éthylène. Les doubles liaisons sont cassées en ajoutant des polymères pour produire de nouvelles liaisons carbone – carbone. Des procédés à haute ou basse pression lors de sa fabrication produiront du PE souple (LDPE) ou du PE dur (HDPE), respectivement (Xie et al., 1994). Très utilisé, le PE est également un des principaux plastiques à se retrouver dans la nature, et sa composition à la structure chimique solide fait du PE un composé persistant et non biodégradable (Vimala and Mathew, 2016; Yang et al., 2014).

Le PP est le second plastique majeur du secteur de l'emballage et correspond à 19.4% de la demande en plastique en Europe (Fig. 1). En plus d'être le composé principal des

contenant micro-ondables, il est le souvent le matériau de choix pour les emballages individuels ou encore de certaines pièces de voiture (Plastic Europe, 2021). Il est obtenu à partir de propylène, suivant les mêmes procédés que le PE, par ajout de monomère de propylène à l'aide catalyseurs. Il possède des propriétés lui conférant une résistance exceptionnelle à la fatigue et faisant donc de lui un matériau de choix pour les objets devant être déformés (Galli, 1994). Tout comme le PE, le PP se retrouve malheureusement souvent dans l'environnement et son taux de biodégradation est très faible (Auta et al., 2018).

Le PVC est le troisième plastique le plus répandu. Il représente 10% de la production de plastique en Europe. Ce dernier est omniprésent dans le secteur du bâtiment au niveau des cadres de fenêtres, divers revêtements du sol et des murs ou encore dans la conception de tuyaux (Plastic Europe, 2021). Tout comme le PE et le PP, c'est un composé aliphatique qui diffère par sa teneur en chlore qui est de l'ordre de 57%. Sa préparation se fait par polymérisation de monomère de chlorure de vinyle obtenu par chloration d'éthylène (Saeki and Emura, 2002; Titow, 2012). C'est un polymère solide et résistant, dont les applications demandent une résistance à la biodégradation (Titow, 2012).

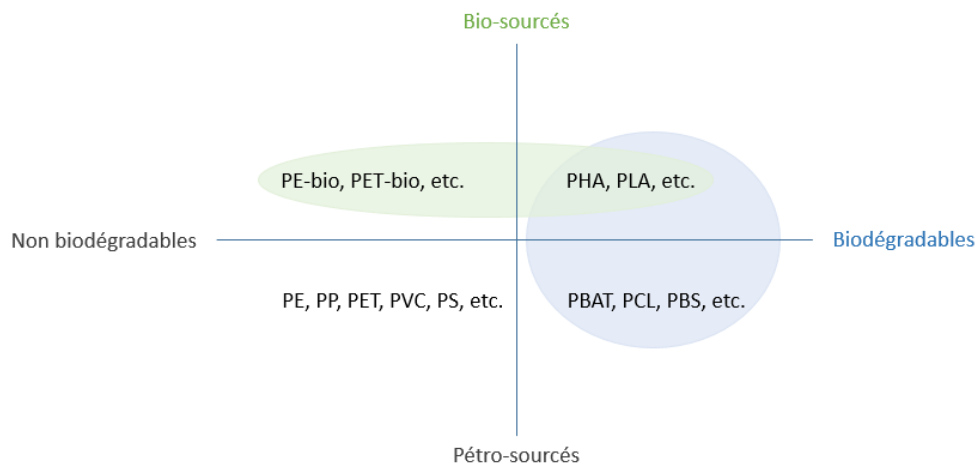
Le PET, quatrième plastique non biodégradable le plus demandé en Europe, est un composé couramment retrouvé dans le secteur de l'emballage, plus particulièrement dans la composition des contenants de boissons (Plastic Europe, 2021). Il est obtenu par condensation entre l'acide téréphtalique et l'éthane- 1,2 –diol suivant une étape d'estérification puis de polycondensation des oligomères (Bruzard, 2021). Chimiquement, le PET possède un groupement aromatique ce qui lui confère une certaine rigidité recherchée dans les bouteilles en plastiques utilisées mondialement aujourd'hui (Bruzard, 2021).

Le PS et PSE (polystyrène expansé), est le polymère le plus utilisé dans le secteur de l'isolation. Efficace, léger et abordable, il est également bien présent dans le secteur de l'emballage alimentaire. Il est le polymère le plus produit après le PET. Obtenu à partir de la polymérisation du styrène, par copolymérisation avec le butadiène ou l'acrylonitrile, ce plastique possèdent un poids moléculaire très élevé ce qui limite très fortement sa biodégradation dans l'environnement (Ho et al., 2018).

## b. Les plastiques dits biodégradables

Les plastiques dits biodégradables sont souvent confondus avec les bioplastiques. Le terme bioplastique correspond plus exactement à trois catégories de plastiques : - les plastiques biosourcés provenant de la biomasse, le plus souvent du maïs, de sucres ou de cellulose, - les

plastiques biodégradables peu importe leur source tant qu'ils peuvent être minéralisés sous forme d'eau et de dioxyde de carbone (ou méthane) selon un temps déterminé et certaines conditions et – les plastiques biosourcés et biodégradables. Un plastique biosourcé peut ne pas être dégradé, c'est le cas du PE biosourcé et du PET biosourcé qui dispose de la même structure chimique que le PE et le PET mais dont la source de fabrication est en partie végétale avant transformation du composé (Fig. 2) (Bruzard, 2021). Inversement, un plastique d'origine pétrochimique peut être biodégradable tel que le poly(butène adipate téréphtalate) (PBAT) ou le poly(succinate de butyle) (PBS) (Fig. 2). Les plastiques biodégradables sont donc régis par un paramètre : celui de la minéralisation des atomes de carbone et d'oxygène du polymère en H<sub>2</sub>O et CO<sub>2</sub> (ou CH<sub>4</sub> en anaérobie) sous l'action de macro- et microorganismes.



**Figure 2 :** Catégorisation des plastiques selon leur source et biodégradabilité

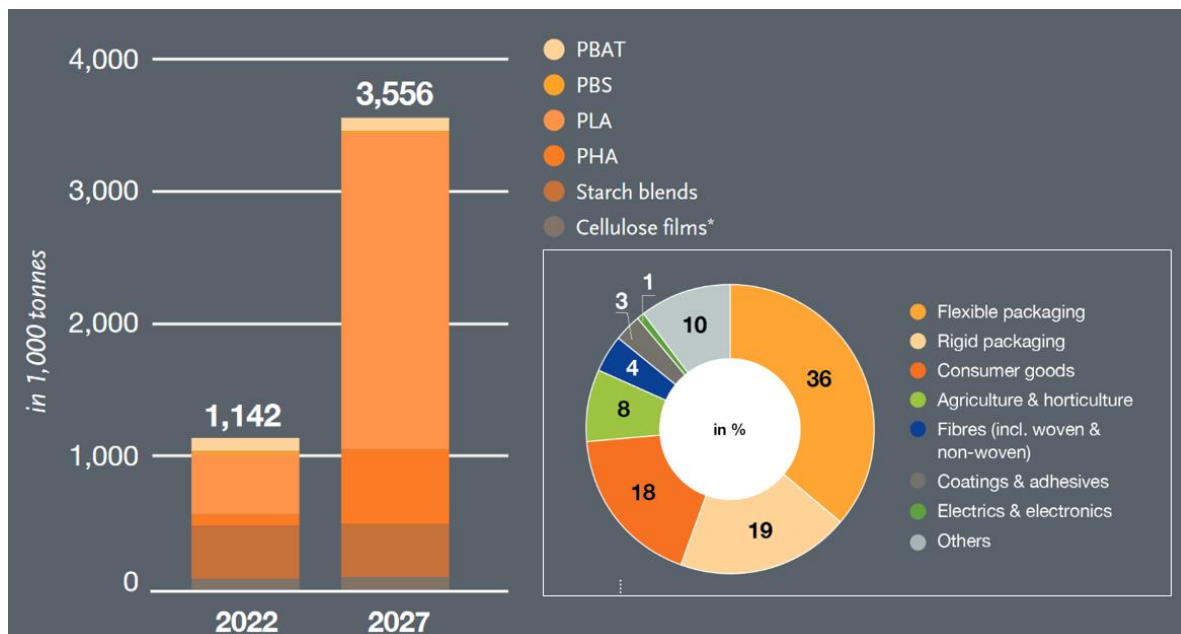
Les plastiques biodégradables les plus connus sont le PBAT, le PBS, la polycaprolactone (PCL), l'acide polylactique (PLA) et les polyhydroxyalcanoates (PHA). En Europe, la production de plastiques biodégradables est faible comparée à celle des plastiques dits conventionnels (1 142 tonnes en 2022) (Fig. 3). Le marché prévoit une augmentation et la production de plastiques biodégradables devrait atteindre les 3 556 tonnes en 2027 (Fig. 3) (Manger, 2022), même si leur production restera toujours inférieure à moins de 1% de la production mondiale de plastiques. L'emballage est actuellement la principale application des plastiques biodégradables, il représente 50% de la production en 2021 en Europe. Suivent les secteurs de l'agriculture avec l'emploi de paillages biodégradables et les biens de consommation qui représentent 14% et 13%, respectivement (Fig. 3) (Manger, 2022).

Le PBAT, polymère aromatique aliphatique, est le plastique biodégradable le plus produit (19.2% en 2021) (Manger, 2022). Obtenu à partir du butane- 1,4 –diol, d'acide hexanedioïque et d'acide téréphtalique, c'est un copolyester alternant motifs rigides d'acide téréphtalique et de motifs souples provenant du butane- 1,4 –diol (Bruzaud, 2021). Ses propriétés font du PBAT un excellent remplaçant au LDPE dans le secteur de l'emballage alimentaire et de l'agriculture (films d'emballage et paillages agricoles). Souvent mixé avec de l'amidon, du maïs ou de la pomme de terre, le PBAT est biodégradable en compost et les films qu'ils composent sont en accord avec la norme NF EN ISO 170033 qui garantit une dégradation d'au moins 90 % après 2 ans d'enfouissement (Bruzaud, 2021).

La production de la PCL est limitée à l'échelle du marché dû à ses faibles applications. Ce polymère est fabriqué à partir de l'acide 6- hydroxyhexanoïque et de l'  $\epsilon$ -caprolactone. La PCL montre une bonne biodégradabilité dans le sol : 70% de la matière enfouie a été minéralisées après 360 jours. Cependant, bien qu'elle fut utilisée autrefois en agriculture, ses propriétés phytotoxiques limitent son usage au domaine médicale, notamment pour la fabrication de fils de sutures résorbables (Bruzaud, 2021).

Le PBS représente 3.5% de la part de marché des plastiques biodégradables en 2021 (Manger, 2022). Il est conçu à partir d'acide succinique et de butane- 1,4 –diol. Tout comme le PBAT, le PBS est principalement utilisé pour l'emballage et les films de paillages bien que ce polymère trouve quelques applications dans le biomédicale ou la cosmétique (Peñas et al., 2022). Bien que souvent mixé avec d'autres polymère, le PBS atteint des taux de dégradation respectant la plupart des normes d'organisations internationales (Peñas et al., 2022).

Le PLA est un polymère aliphatique bio-sourcé, c'est-à-dire qu'il est issu de sucre de betterave ou de maïs. Sa synthèse se fait par condensation de l'acide lactique ou bien par polymérisation par ouverture de cycle (Bruzaud, 2021; Trivedi et al., 2023). Ce polymère est biodégradé totalement dans des conditions particulières de compostage à 60°C (Castro-Aguirre et al., 2018). En tant que thermoplastique, ces applications sont nombreuses que ce soit dans le biomédical, l'emballage alimentaire mais aussi des sacs de compostage pour les biodéchets (Farah et al., 2016).



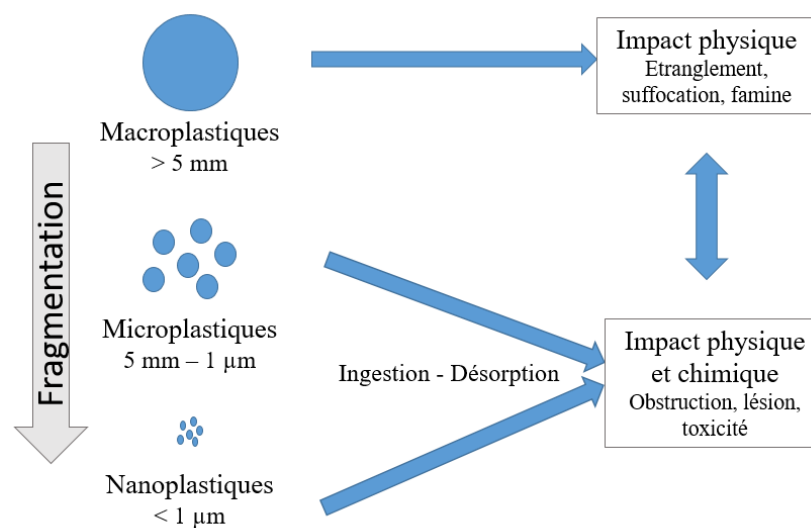
**Figure 3 :** Production de plastiques biodégradables en 2022 et prévision du marché en 2027 et potentiels secteurs d'applications (Manger, 2022)

Les PHA sont des polyesters aliphatiques obtenues par fermentation bactérienne à partir de différents substrats carbonés. Ce type de polymère sera décrit en section 3.

### 1.3 La pollution plastique des océans

Les océans et les mers constituent un grand réservoir de déchets plastiques. Arrivés du continent, la pollution plastique correspond à environ 45 000 tonnes dispatchés dans tous les océans y compris les zones polaires (Ghiglione and Dussud, 2014). Des zones d'accumulations créées par les courants marins forment des gyres océaniques où les quantités de plastiques peuvent atteindre 129 000 tonnes (Lebreton et al., 2012). A la surface de la mer, les principaux débris plastiques retrouvés sont composés de PE, suivi du PP et du PS (Auta et al., 2017). Sous forme de macroplastiques (> 5 mm), cette pollution affecte particulièrement la faune et la flore marine par suffocation, famine, étranglement ou ingestion (Fig. 4) (Browne et al., 2007; Harrison et al., 2011). Ces macroplastiques vont se fragmenter sous l'effet des UV, des vagues ou encore de la colonisation de divers organismes et vont devenir des microplastiques (entre 5 mm et 1 µm) puis des nanoplastiques (< 1 µm) (Fig. 4). Ils peuvent entraîner une obstruction ou une lésion des organes des organismes qui les ingèrent (GESAMP, 2015) (Fig. 4). En effet, ces plastiques contiennent de nombreux contaminants chimiques provenant de la structure chimique même du plastique ou de contaminants absorbés au cours de son cycle de vie mais surtout de l'ajout d'additifs lors de sa conception (Rochman, 2015). Ces contaminants

organiques tels que des polychlorobiphényles (PCB), les polybromodiphényléthers (PBDEs), bisphenol A (BPA) ou encore des hydrocarbures aromatiques polycycliques (HAP) présentent une toxicité élevée, sont persistants dans l'environnement et le plus souvent bioaccumulatifs (Rochman, 2015) (liste non exhaustive). En plus d'affecter la faune et la flore à travers des phénomènes de désorption et de toxicité chimique (fig. 4), certains de ces polluants ont une influence sur les communautés microbiennes et leur fonctionnement (Lear et al., 2021).



**Figure 4 :** Schéma des différents impacts des déchets plastiques retrouvés en mer en fonction de leur taille. Inspiré de Harrison et al., 2011.

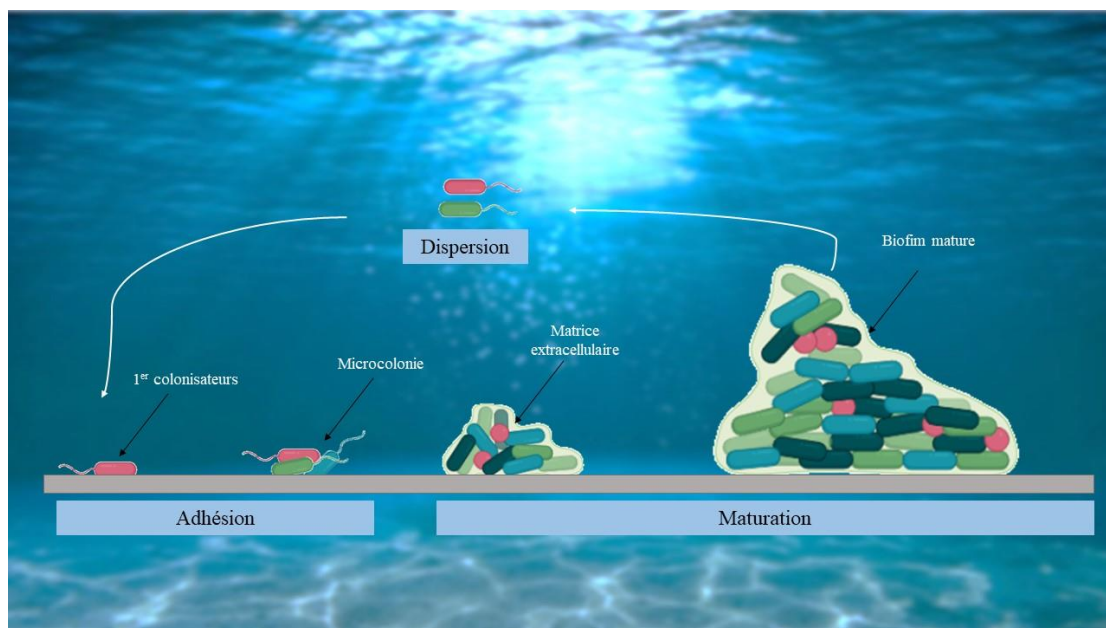
## 2 La biodégradation de plastique en mer

### 2.1 Le biofilm

Dans le milieu marin, les organismes microbiens se développent rapidement à la surface des plastiques immergés, ils composent un biofilm. Ce biofilm est le résultat du développement d'une communauté multicellulaire pouvant être composée de bactéries, champignons ou algues adhérant à la surface solide à l'aide d'une matrice constituée de sécrétions adhésives et protectrices (Costerton et al., 1987). Cette matrice a une structure tridimensionnelle et est principalement composée d'exopolysaccharides (EPS), de lipides et d'ADN extracellulaire (Flemming and Wingender, 2010). Elle peut constituer plus de 90 % de la masse sèche du biofilm et permet la capture par sorption passive de diverses ressources telles que des nutriments, des gaz ou autre molécules organiques nécessaires aux organismes

(Almstrand et al., 2013). La structure complexe du biofilm fournit donc plusieurs avantages incluant la protection des prédateurs, une meilleure tolérance à la dessiccation ou encore à certaines résistances aux antibiotiques (Flemming et al., 2016).

La formation d'un biofilm se réalise en plusieurs étapes (Fig. 5). La première phase de primo-colonisation correspond à la fixation des cellules à la surface grâce à des mécanismes impliqués dans la détection de surface. Une cascade de signalisation responsable de l'adhérence cellulaire se met en place favorisée par la présence de pili, de fimbriae, de flagelle de type IV ou de polysaccharides associés aux microorganismes adhérents (Donlan, 2002; O'Toole and Wong, 2016). La phase de croissance se définit par la formation d'une microcolonie dans laquelle des signaux chimiques d'intercommunications sont émis lors de la multiplication des bactéries, ce qui engendre le principal composant de la matrice : les exopolysaccharides (EPS). Le biofilm entre ensuite dans une troisième phase de maturation qui correspond à une stabilisation de l'utilisation de la surface du plastique et du grossissement du biofilm. C'est également une phase de dispersion qui se met en place, où certaines bactéries sont libérées dans le milieu pour aller coloniser d'autres surfaces (Maunder and Welch, 2017).



**Figure 5** : Schéma de la formation d'un biofilm sur la surface d'un matériau colonisé en milieu marin

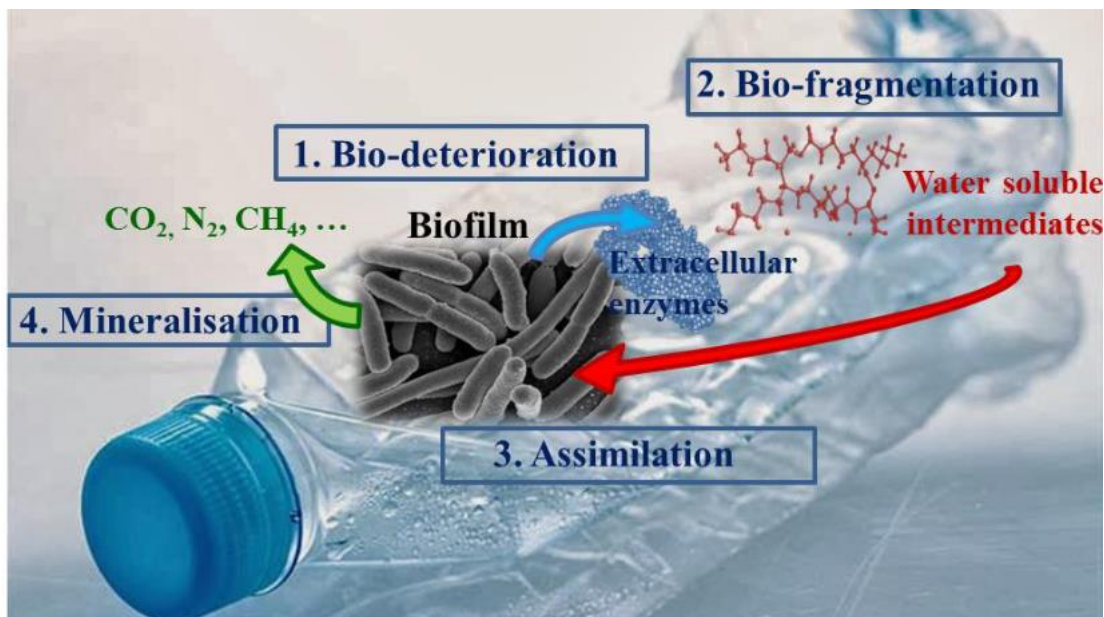
## 2.2 Mécanisme de biodégradation

La biodégradation est un processus biologique qui est concomitant à un processus « abiotique », où la structure des polymères va être altérée à travers des phénomènes physiques (vagues, température et rayons UV) et chimiques (hydrolyses ou oxydation) (Shah et al., 2008).



La dégradation biotique, impliquant les microorganismes se déroule par la suite en 4 étapes décrites par Dussud et Ghiglione, (2014) (Ghiglione and Dussud, 2014) (Fig. 6).

La première étape dite de « bio-détérioration » correspond à la fois aux modifications physiques du plastique causées par la formation du biofilm qui provoque ou accentue les fissures, mais aussi à des modifications chimiques suite à la libération de composés acides au sein de la structure. La seconde étape dite de « bio-fragmentation » se déroule à travers l'action d'enzymes extracellulaires telles que des lipases, des estérases ou des dépolymérase libérées par les microorganismes d'un biofilm mature. Ces enzymes provoquent la libération d'oligomères et de monomères dans le milieu environnant, réduisant le poids moléculaire du plastique. Ces oligomères et monomères libérés atteignent une taille inférieure à 600 Daltons et sont solubles dans leur milieu. Les bactéries vont pouvoir les assimiler en les transportant à travers leur membrane cellulaire constituant la troisième étape : la « bio-assimilation ». L'ultime étape de la biodégradation correspond à la minéralisation de ces composés solubles, ou « bio-minéralisation ». A travers leur métabolisme, les bactéries vont transformer les oligomères et monomères en métabolites oxydés ( $\text{CO}_2$ ,  $\text{N}_2$ ,  $\text{CH}_4$  et  $\text{H}_2\text{O}$ ).



**Figure 6 :** Etapes de la biodégradation du plastique en mer (selon Ghiglione and Dussud, 2014)

### 2.3 La plastisphère

Le terme « plastisphère » introduit la vie microbienne sur le plastique mise en évidence récemment par des travaux utilisant des techniques modernes de séquençage massif d'ADN (Zettler et al., 2013). De nombreux microorganismes incluant des bactéries, des archées, des

champignons et des eucaryotes microbiens ont été détectés dans plusieurs études, à partir de plastiques prélevés en mer ou incubés expérimentalement dans des conditions marines (Jacquin et al., 2019). La différence entre les bactéries vivant sur les plastiques et les bactéries vivant à l'état libre ou sur les particules organiques de l'eau de mer a été soulignée par plusieurs études quel que soit le type de plastique étudié, soulignant que le plastique est une nouvelle niche écologique (Debroas et al., 2017; C. Dussud et al., 2018; Oberbeckmann et al., 2017; Zettler et al., 2013).

Le type de polymère, la localisation géographique, les variations saisonnières ainsi que le type de plastiques influencent la composition des communautés microbiennes de la plastisphère (Basili et al., 2020; Oberbeckmann et al., 2016). Elle constitue une niche écologique diverse et variée favorisant le transfert horizontal de gènes, la compétition et les interactions microbiennes (Flemming et al., 2016; Sørensen et al., 2005). Sa composition varie donc également au cours du temps dans un même environnement donné (De Tender et al., 2017; Claire Dussud et al., 2018; Odobel et al., 2021). Lors de la phase de primo-colonisation, les Gammaproteobacteria sont majoritaires (De Tender et al., 2017; Claire Dussud et al., 2018; Harrison et al., 2014) et laissent place au Alphaproteobacteria et Bacteroidetes lorsque le biofilm devient mature (Claire Dussud et al., 2018; Lee et al., 2008; Odobel et al., 2021). Bien que les propriétés propres au type de polymère telles que l'hydrophobicité, la cristallinité ou la rugosité peuvent influencer la composition des communautés bactériennes, ces dernières semblent plutôt dépendre du processus interne de mise en place du biofilm lorsqu'il atteint sa maturité au environ d'un mois d'immersion (Claire Dussud et al., 2018; Odobel et al., 2021; Pompilio et al., 2008). Certaines familles de bactéries telles que les Rhodobacteraceae, connues pour coloniser diverses surfaces sont souvent retrouvées sur plusieurs types de plastique (Claire Dussud et al., 2018; Elifantz et al., 2013; Moura et al., 2018; Oberbeckmann and Labrenz, 2020; Odobel et al., 2021). A l'exception de quelques familles comme les Cryomorphaceae et Alcanivoraceae (Oberbeckmann et al., 2016) qui semblent être spécifique du PET, les plastiques sont comme une surface disponible à l'attachement d'un biofilm plutôt qu'un outil de sélection bactérien (Kirstein et al., 2018).

Le biofilm peut contenir des bactéries capables de dégrader certains polymères (Barnes, 2002; Debroas et al., 2017; Zettler et al., 2013). Avec une surexpression de la voie métabolique impliquée dans la dégradation des xénobiotiques, les bactéries observées sur les plastiques ont un métabolisme différent des micro-organismes vivant dans l'eau environnante (Bryant et al., 2016; Debroas et al., 2017; Odobel et al., 2021). En ce qui concerne les PHA, des gènes codant

pour des dépolymérase spécifiques (par exemple, la polyhydroxybutyrate dépolymérase), l'estérase ou l'adénylyl sulfate réductase, ont été retrouvés dans des communautés microbiennes attachées à leur surface (Pinnell and Turner, 2019). Cependant, les communautés microbiennes vivant sur le PHBV semblent être plus actives et spécifiques que les bactéries sur les plastiques non biodégradables, et une succession de dégradeurs de PHA a été observée au cours des phases précoces du biofilm, de sa croissance et de la maturation (Claire Dussud et al., 2018). Il a également été démontré sur ce même PHA mais aussi d'autres plastiques biodégradables (PLA, PHBV et PCL) qu'une quantité supérieure d'enzymes hydrolytiques telles que la protéase, la lipase et la glucosidase est présente dans le biofilm par rapport à la plastisphère de polymères non biodégradables (Odobel et al., 2021).

## 2.4 Méthode d'étude de la plastisphère

La composition et les fonctions des communautés microbiennes peuvent être étudiées à travers différentes approches « omiques ». Ces méthodes regroupent les analyses métagénomiques (l'étude des gènes), métaprotéomiques (l'étude des protéines) et métatranscriptomiques (l'étude du transcriptome). Elles couvrent une large partie des communautés microbiennes et permettent d'éviter les biais rencontrés en culture de laboratoire en mettant également en lumière les microorganismes non-cultivables (Morales and Holben, 2011). Le séquençage d'une partie du gene de l'ADNr 16S (métabarcoding) permet d'étudier la diversité taxonomique et la structure d'une communauté microbienne (Poretsky et al., 2014). Bien que moins précis que l'étude de l'ensemble des gènes, le métabarcoding est peu coûteux, rapide et amène une résolution suffisante pour l'identification taxonomique au niveau du genre et parfois de l'espèce (Poretsky et al., 2014).

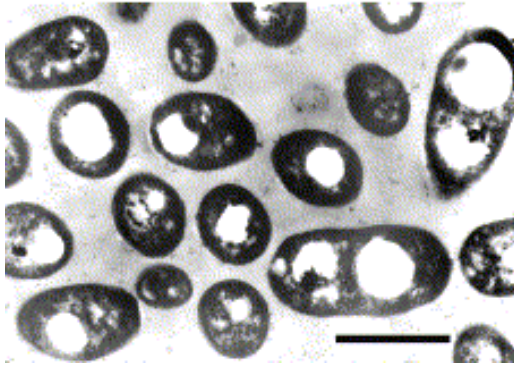
Des travaux ont montré que les techniques classiques de mesures d'activités enzymatiques bactériennes étaient adaptables à la plastisphère. Des mesures colorimétriques d'activités ectoenzymatiques (lipases, glucosidases, aminopeptidases) ont été mesurées sur des plastiques (Odobel et al., 2021), ainsi que des mesures d'activité hétérotrophe bactérienne (Claire Dussud et al., 2018) et de production primaire (Conan et al., 2022). Des techniques de respirométrie sont également utilisables, telles que la mesure de consommation d'oxygène (Sashiwa et al., 2018) ou de production de CO<sub>2</sub> (Deroiné et al., 2015) ou même de production d'ATP (Fontanella et al., 2010). Si toutes ces techniques permettent de mesurer l'impact des plastiques sur les activités microbiennes, elles sont également adaptées pour la mesure de l'activité de la biodégradation microbienne des plastiques.

D'autres méthodes permettent de mesurer l'abondance bactérienne, qui permet notamment de rapporter les mesures d'activités (y compris les activités de biodégradation) au nombre de cellules d'un échantillon. Malheureusement, les dénombrements ont un succès mitigé en milieu naturel. La complexité du biofilm et la nature du plastique limite la mesure en microscopie optique par épifluorescence (Claire Dussud et al., 2018) alors que les méthodes de microscopie électronique (TEM ou SEM), microscopie à force atomique ou microscopie confocale renseignent sur la structure du biofilm mais ne permettent pas un dénombrement microbien direct. La méthode de dénombrement par unité formant colonie (UFC) n'est pas adaptée face à la grande diversité de microorganismes qui colonisent les plastiques en milieu naturel et leur faible taux de cultures (< 1%) (Hugenholtz et al., 2009). La méthode de cytométrie en flux classiquement utilisée en microbiologie marine est possible, mais rencontre également des limites liées au nécessaire détachement du biofilm qui peut être aléatoire en fonction du degré de maturation du biofilm et de sa composition (Claire Dussud et al., 2018). Enfin, la méthode de PCR quantitative également utilisée pour le dénombrement bactérien est possible, mais n'a pas encore été publiée (travaux en cours par l'équipe LOMIC et Plastic@Sea).

### 3 Les PHA, des polymères biosourcés et « biodégradables »

#### 3.1 Généralités

Les PHA sont des composés insolubles dans l'eau qui s'accumulent dans le cytoplasme de certaines bactéries ou d'autres organismes tels que les levures, champignons ou plantes. Ils se présentent sous forme de granules stockés dans le cytoplasme sous forme d'inclusions sphériques (Fig. 7) (Luengo et al., 2003). Ces granules sont composées d'un cœur en polyester amorphe entourée d'une couche de phospholipides entrelacées de diverses protéines (PHA synthases, phasines, et protéines régulatrices) (Grage et al., 2009). Leur rôle biologique est en premier lieu une réserve de carbone afin d'améliorer la survie de l'organisme producteur de PHA lorsque ce dernier subit des changements nutritifs dans son environnement (Kadouri et al., 2005). Les PHA servent également à augmenter diverses résistances aux changements environnementaux comme au stress oxydatif, au choc osmotique ou encore aux températures extrêmes (Goh et al., 2014; Obruca et al., 2020; Soto et al., 2012; Wu et al., 2011; Yin et al., 2015). Découverts pour la première fois en 1926 par Lemoigne dans des cellules de *Bacillus megaterium*, à ce jour, plus de 300 bactéries appartenant à différents genre (Bacillus,



Cupriavidus, Actinomyceres, Streptomyces, Halomonas, Pseudomonas) ont été identifiées comme productrice de PHA (Singh et al., 2018).

**Figure 7 :** Image de granules de PHA dans des cellules de *Pseudomonas putida U* obtenue par microscopie à transmission (Luengo et al., 2003)

### 3.2 Structure chimique et types de PHA

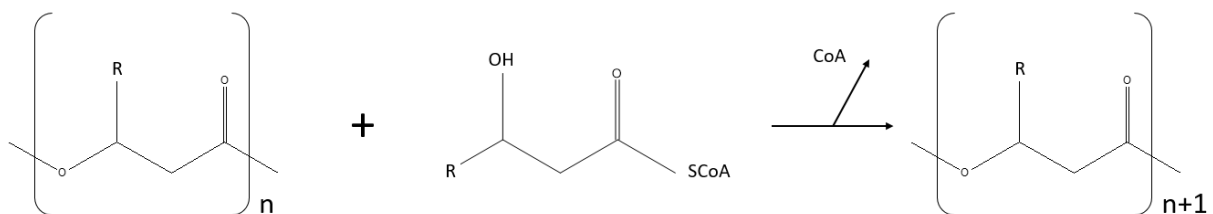
Les PHA sont des polyesters aliphatiques composés de monomères hydroxylés au niveau du troisième carbone asymétrique (Fig. 8), aussi appelée chaîne radicale (R). Ces monomères varient en taille en fonction du nombre de carbone qui compose la chaîne radicale. Deux types de PHA sont distingués en fonction de la taille de cette chaîne radicale : les *short chain length*-PHA (*scl*-PHA) dont la chaîne radicale contient 1 à 2 atomes de carbones et les *medium chain length*-PHA (*mcl*-PHA) lorsque la chaîne radicale dispose de 3 ou un nombre supérieur d'atomes de carbone (Fig. 8). Dès 2010, plus de 150 types de monomères avaient déjà été identifiés (Koller et al., 2010). Les PHA peuvent être sous forme d'homopolymère, c'est-à-dire, constitué d'un seul même type de monomère. C'est le cas du poly-(3-hydroxybutyrate) (PHB), le PHA le plus répandu et le plus étudié (Koller et al., 2010). Ils sont le plus souvent sous forme de copolymères, composés d'au moins deux type de monomères. C'est le cas de tous les *mcl*-PHA dû aux voies métaboliques des bactéries impliquées dans leur synthèse (détaillée en section 3.3 Production de PHA : voies métaboliques) mais aussi du poly-(3-hydroxybutyrate-co-3-hydroxyvalérate) (PHBHV). La structure d'un PHA et la nature de la chaîne radicale dépend de la souche bactérienne et de la source de carbone utilisée mais aussi du procédé de production.

Type de PHA	R	Nom du monomère
<i>scl</i> -PHA	CH <sub>3</sub>	3-hydroxybutyrate (3HB)
	C <sub>2</sub> H <sub>5</sub>	3-hydroxyvalérate (3HV)
<i>mcl</i> -PHA	C <sub>3</sub> H <sub>7</sub>	3-hydroxyhexanoate (3HHx)
	C <sub>4</sub> H <sub>9</sub>	3-hydroheptanoate (3HHp)
	C <sub>5</sub> H <sub>11</sub>	3-hydroxyoctanoate (3HO)
	C <sub>6</sub> H <sub>13</sub>	3-hydroxynonanoate (3HN)
	C <sub>7</sub> H <sub>15</sub>	3-hydroxydecanoate (3HD)
	C <sub>8</sub> H <sub>17</sub>	3-hydroxyundecanoate (3HU)
	C <sub>9</sub> H <sub>19</sub>	3-hydroxydodecanoate (3HDD)

**Figure 8 :** Structure générale d'un PHA et nomenclature des monomères selon le nombre d'atomes de carbone associée à la chaîne radicale. n représente le nombre de monomère et R la longueur de la chaîne radicale.

### 3.3 Production de PHA : voies métaboliques

Le type de PHA accumulé dépend de la souche bactérienne et du substrat utilisés mais plus spécifiquement de la PHA synthase (PhaC). Enzyme essentiel à la polymérisation des monomères de PHA (Fig. 9) en catalysant la conversion de (R)-3-hydroxyacyl-CoA en PHA, il en existe 4 classes classifiées en fonction de la structure des gènes codant, leur spécificité de substrat et leur composition en sous-unité ou non (Rehm, 2003).



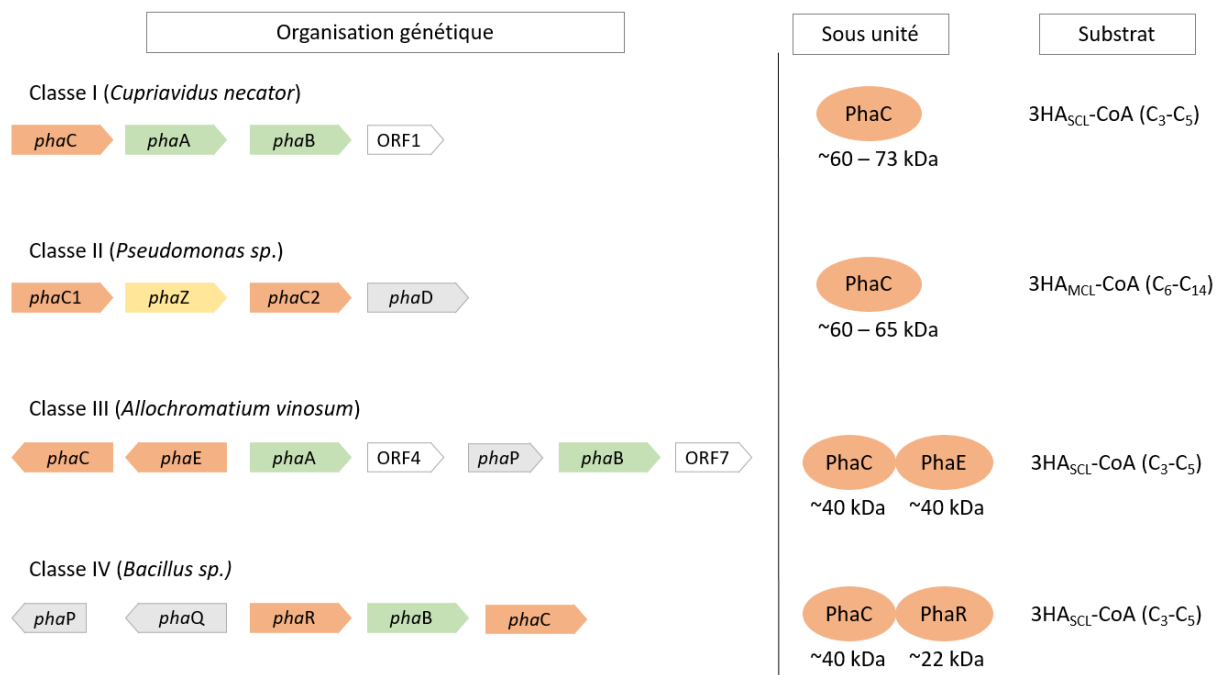
**Figure 9 :** Réaction de polymérisation des monomères de PHA par la PHA synthase (Rehm, 2003). n représente le nombre de monomère et R la longueur de la chaîne radicale.

La PHA synthase de classe I, bien étudiée chez *Cupriavidus necator* est codée par le gène phaC et composée d'une unité de 61 à 73 kDa. Elle est principalement active sur des substrats composés de 3 à 5 atomes de carbone (Fig. 10) (Sudesh et al., 2000) mais son affinité

pour une large variété de substrat lui permet également d'incorporer certains monomères appartenant aux *mcl*-PHA (Brandl et al., 1989; Fukui and Doi, 1997). Les PHA synthases de classe II sont typiquement retrouvées dans les bactéries du genre *Pseudomonas* et incorporent les substrats composés de 6 à 14 atomes de carbones (Fig. 10) (Rehm, 2003; Sudesh et al., 2000). Leur taille est relativement similaire aux PHA synthases de classe I (entre 60 et 65 kDa). La particularité des PHA synthases de classe III et IV est leur structure composée de deux sous-unités. La PHA synthase de type III contient une sous unité C d'environ 40 kDa et une autre sous unité E d'environ 40 kDa. La présence de ces deux sous-unités n'est pas indispensable au fonctionnement de l'enzyme mais son activité est fortement inférieure (< 1%) en l'absence de la sous-unité E (Rehm, 2003). L'organisme le plus connu possédant ce type de PHA synthase est *Allochromatium vinosum* (anciennement *Chromatium vinosium*) et ce type d'enzyme possède une forte affinité pour les substrats composés de 3 à 5 atomes de carbone (Fig. 10) (Liebergesell and Steinbüchel, 1992).

Les PHA synthases appartenant à la classe IV ont une structure similaire à celle retrouvée dans les classes III. La PhaC principale fait environ 40 kDa et est complétée par une sous-unité R d'environ 22 kDa. Tout comme PhaE, PhaR est nécessaire pour atteindre une activité enzymatique efficace. Bien que PhaE et PhaR ne partagent que 18% de similarité dans leur séquence d'acides aminés, leur rôle semble similaire dans le remplacement de l'extrémité C-terminale hydrophobe présente dans les PHA synthases de classe I et II. Cette extrémité hydrophobe assure le contact avec le noyau hydrophobe du polyester (Rehm, 2003). Cette PHA synthase de classe IV a été principalement étudiée chez *Megacillus megaterium* et a une affinité de substrat pour les monomères composés de 3 à 5 atomes de carbones (Fig. 10).

Bien que ce ne soit pas une généralité, les gènes codant pour les PHA synthases sont souvent organisés en opéron facilitant la modification génétique d'organismes pour augmenter les rendements de production. Ces modifications concernent le plus souvent l'ajout de PHA synthase de classe II dans des organismes pouvant faire des *scl*-PHA afin d'obtenir des copolymères de *scl*- et *mcl*-PHA mais certaines modifications génétiques ciblent également la  $\beta$ -oxydation afin d'augmenter le rendement en *mcl*-PHA (Silva et al., 2021).

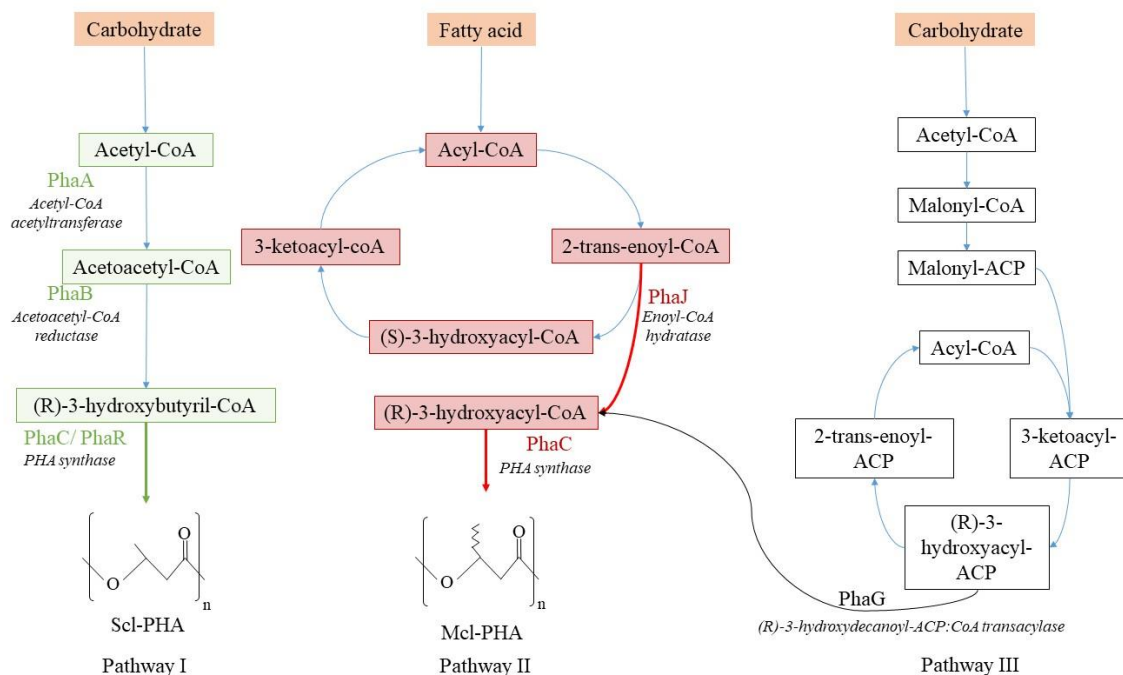


**Figure 10:** Organisation génétique des quatre classes de PHA synthases, leur structure et leur affinité de substrat adaptée de Rehm, 2003; Sudesh et al., 2000. phaC/C1/C2 : gènes codant pour la PHA synthase ; phaE : gène codant pour la sous-unité de la PHA synthase de classe III ; phaA : gène codant pour l'acetyl-CoA acetyltransferase ; phaB : gène codant pour l'acetoacetyl-CoA-reductase ; phaR : gène codant pour la sous-unité de la PHA synthase de classe IV ; phaZ : gène codant pour la PHA depolymérase ; autres gènes : fonctions non détaillées.

Ces enzymes interviennent dans la biosynthèse de PHA à travers trois voies métaboliques distinctes et dites naturelles (sans ingénierie génétique). Ces voies métaboliques conduisent à différents types de PHA (*scl*- ou *mcl*-PHA) et dépendent de la source de carbone utilisée en tant que substrat mais aussi de la souche et de la PHA synthase impliquée.

La première voie (pathway I, Fig. 11) est la voie utilisée pour la synthèse de *scl*-PHA à partir de sucres. Après l'obtention d'acetyl-CoA à partir de la glycolyse du sucre, l'acetyl-CoA acetyltransferase (PhaA) va le condenser en acetoacetyl-CoA. L'acetoacetyl-CoA reductase (PhaB) va réduire cet acetoacetyl-CoA en (R)-3-hydroxybutyryl-CoA. Ce dernier est enfin disponible et converti en *scl*-PHA par les différentes PHA synthases (PhaC ou PhaC / PhaE ou PhaC / PhaR) (Sudesh et al., 2000).





**Figure 11:** Voies métaboliques de la synthèse de *scl*- et *mcl*-PHA inspiré de Prieto et al., 2016; Sudesh et al., 2000; Zinn, 2010

La deuxième voie est la voie de la  $\beta$ -oxydation (Pathway II, Fig. 11). Elle est la voie principale de biosynthèse des *mcl*-PHA. Les acides gras entrent dans le cycle de la  $\beta$ -oxydation sous forme d'acyl-CoA puis de 2-trans-enoyl-CoA. L'enzyme enoyl-CoA hydratase (PhaJ) dérouté le composé du cycle de la  $\beta$ -oxydation pour assurer la transformation du 2-trans-enoyl-CoA en stéréoisomère (R)-3-hydroxyacyl-CoA. La PHA synthase de classe II converti ce dernier en *mcl*-PHA (Zinn, 2010). Les acides gras sont aussi appelés substrats reliés au PHA produit. C'est-à-dire que la composition en monomère des *mcl*-PHA sera identique au nombre de carbone du substrat ou bien réduite de 2, 4 ou même 6 atomes de carbones selon l'affinité de l'enzyme pour le substrat (Anjum et al., 2016). Ainsi, un *mcl*-PHA produit à partir d'acide nonanoïque pourra avoir une composition molaire de 60% de HN et 40% de HHp (Sun et al., 2009).

La synthèse de novo des acides gras est une voie indirecte de production de *mcl*-PHA à partir de sucres (Pathway III, Fig. 11). A travers la synthèse des acides gras, les sucres vont être transformé en (R)-3-hydroxyacyl carrier protein (ACP). La (R)-3-hydroxydecanoyl-ACP-CoA transacylase (PhaG) peut intervenir dans ce cycle pour convertir le (R)-3-hydroxy-ACP en (R)-

3-hydroxyacyl-CoA disponible pour la PHA synthase spécifique au *mcl*-PHA (Prieto et al., 2016; Sudesh et al., 2000; Zinn, 2010).

### 3.4 Propriétés et applications

Les propriétés physico-chimiques diffèrent entre les *scl*-PHA, qui sont des polymères rigides et cassants, et les *mcl*-PHA qui sont généralement plus caoutchouteux et ductiles (Pérez-Rivero and Hernandez-Raquet, 2017). Le poly(3-hydroxybutyrate) (PHB) est l'un des PHA les plus répandus et le mieux caractérisé. Avec une cristallinité élevée (> 50 %), c'est un polymère relativement cassant et rigide (Corre et al., 2012; Koller et al., 2010). L'incorporation de 3-hydroxyvalérate (HV) forme un copolymère poly(3-hydroxybutyrate-co-3-hydroxyvalérate) (PHBHV) moins rigide et cassant, plus facile à traiter pour des applications commerciales (Lemechko et al., 2019; Pachekoski et al., 2009). Les *mcl*-PHA affichent des propriétés qui pourraient remplacer les élastomères. Ils sont caoutchouteux, mous et présentent un faible degré de cristallinité et des températures de fusion et de transition vitreuse plus basses (Abe et al., 2012). Le tableau 1 résume certaines propriétés thermiques et mécaniques des PHA les plus produits et/ou plus étudiés (Abe et al., 2012; Koller et al., 2010; Singh et al., 2018). En fonction de la longueur de la chaîne radicale ou de sa composition, les propriétés varient grandement entre les *scl*- et *mcl*-PHA mais également au sein des *scl*-PHA en fonction du pourcentage d'incorporation en HV.

La versatilité des propriétés des PHA donne à ces polymères des applications variées. On retrouve principalement des *scl*-PHA puisqu'il n'existe pas de véritable *mcl*-PHA en vente (voir 3.5 Marché des PHA). Les PHA pourraient trouver des applications dans le domaine de l'emballage ou dans la conception de consommables (couverts, assiettes etc) (Bugnicourt et al., 2014; Philip et al., 2007), dans le domaine de l'agriculture pour la fabrication de paillages ou filets (Amelia et al., 2019; Philip et al., 2007) ou encore dans le domaine du biomédical ou des nanotechnologies (Philip et al., 2007; Zhang et al., 2018).

**Tableau 1 :** Certaines propriétés thermiques et mécaniques de plusieurs PHA (Abe et al., 2012; Koller et al., 2010; Singh et al., 2018)

Polymer	Propriétés thermiques		Propriétés mécaniques	
	Température de fusion (°C)	Transition vitreuse (°C)	Résistance à la traction (Mpa)	Elongation (%)
PP	170	-10	38	400
PHB	~180	0 à 10	20 à 40	0.4 à 6
PHBHV 8%	165	1	19	35
PHBHV 20%	145	-1	32	30 à 50
PHBHV 70%	87	-13	-	
PHO*	51	-47	4,3	162
PHN*	~56	~-45	2,6	184
PHD*	~67	~-50	2,8	171

\*Copolymères de *mcl*-PHA majoritairement composé de HO, HN ou HD

### 3.5 Marché des PHA

Le marché des PHA se concentrent essentiellement sur les *scl*-PHA et représente 3.9% des capacités de production des bioplastiques en 2022 (European Bioplastics, nova-institute, 2022). Plusieurs entreprises proposent des PHA avec des capacités de production variant de 100 à 10 000 tonnes par an (Tableau 2). La source de carbone utilisée lors de la production de PHA représente un coût considérable, presque 50% du coût total du matériau (Ciesielski et al., 2015). Les entreprises assurant une production industrielle des PHA utilisent majoritairement des substrats d'origine végétale tel que le maïs, la canne à sucre ou divers huiles végétales afin de réduire les coûts de production. La principale souche utilisée industriellement est *Cupriavidus necator*, modifié génétiquement ou non en fonction de la composition du PHA recherchée (Tableau 2). Un seul PHA composé de *mcl*-PHA est disponible sur le marché, il s'agit du Nodax qui contient au minimum 3% de monomères de HHx et commercialisé par Danimer Scientific (Tableau 2).

**Tableau 2:** Principales entreprises vendant des PHA et leur spécificité (Koller and Mukherjee, 2022) (liste non exhaustive).

Entreprise	Strain	PHA produit	Substrat	Usage revendiqué	Capacité de production (Tonnes/an)
Danimer Scientific	<i>Aeromonas caviae</i> ou <i>Aeromonas hydrophila</i> ou <i>Cupriavidus necator</i> recombinée	Nodax (PHBHHx)	Huile de canola	Résines pour emballages alimentaires	3500
Newlight Technologies	Marine bacteria	PHB	Méthane	Biomédical	
Tianan Biologic Materials	<i>Cupriavidus necator</i>	PHBHV	Maïs	Multiple	2000
Bio-On	<i>Cupriavidus necator</i>	PHB, PHBHV	Canne à sucre	Médical et bioremédiation	~2000
Kaneka Corporation	<i>Cupriavidus necator</i> recombinée	PHBHV	Huiles végétales	Emballage alimentaire	n.r
Shenzhen Ecomann Biotechnology Co., Ltd	n.r	PHA	Glucose, maïs	Sacs et films plastiques	100
Tianjin GreenBio Materials Co., Ltd	<i>Escherichia coli</i> recombinée	PHBHV	Glucose, 1,4-butanediol	Films et mousses plastiques	10 000
Biomer	<i>Azohydromonas australica</i>	PHB	Sucrose	Fournisseur de granules de PHB	900
Biocycle	<i>Paraburkholderia sacchari</i>	PHB	Sucrose	Emballage alimentaire	100
RWDC industries	<i>Cupriavidus necator</i>	PHBHV	Déchet d'huiles de cuisson	Médical et bioremédiation	1000

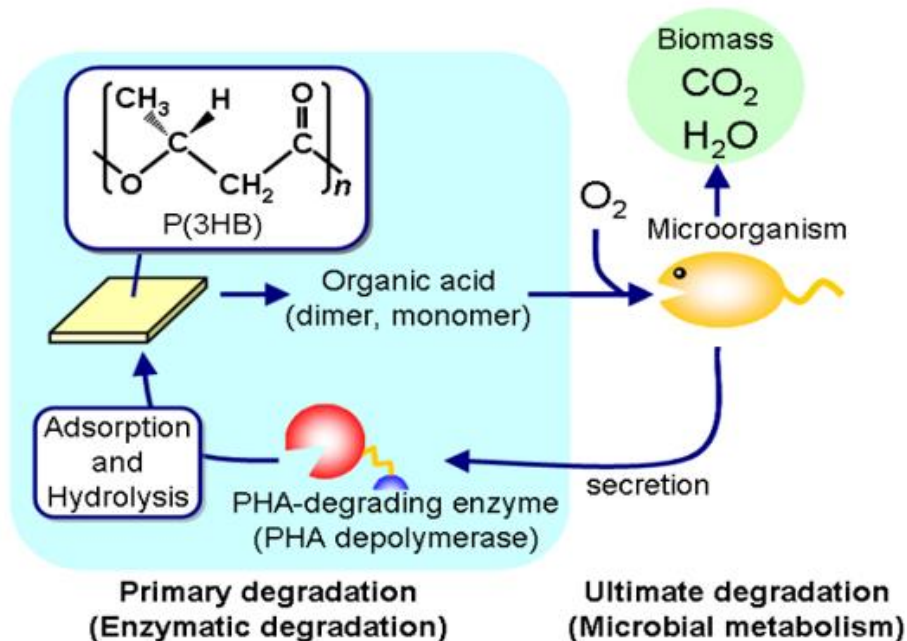
### 3.6 Biodégradation des PHA : voies métaboliques

Les PHA, en particulier les *scl*-PHA, ont montré des biodégradabilités en milieu marin supérieures à la cellulose (Deroiné et al., 2014; Volant et al., 2022). Certains micro-organismes sont dotés d'enzymes spécialisées dans la dépolymérisation des PHA, les PHA dépolymérase (PhaZ) (Knoll et al., 2009). On distingue, en premier lieu, deux types de PHA dépolymérase :

la PHA depolymerase intracellulaire (nPhaZ pour « natured » PHA depolymerase) et la PHA depolymerase extracellulaire (dPhaZ pour « denatured » PHA depolymerase).

La PHA depolymerase intracellulaire permet l'hydrolyse des granules de PHA accumulées dans le cytoplasme (Doi et al., 1990). Elle est active lorsque la bactérie se retrouve dans un environnement pauvre en carbone. Le PHA, sous forme natif et amorphe, est dépolymérisé par l'enzyme, apportant carbone et énergie au métabolisme bactérien. Cette enzyme intracellulaire, ne concerne que la dégradation des PHA internes à la cellule, et n'est donc pas active sur les PHA libres dans l'environnement (Sznajder and Jendrossek, 2011).

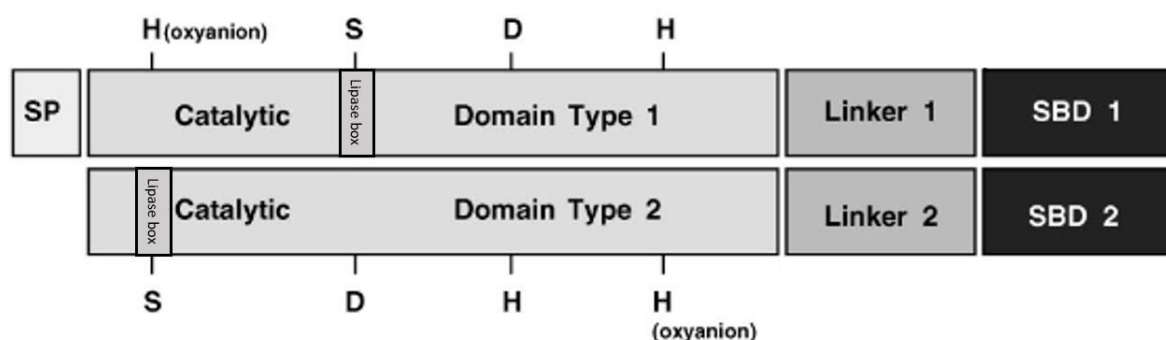
Dans l'environnement naturel, la biodégradation des PHA est principalement due aux PHA dépolymérase externes (Brandt et al., 1995; Pinnell and Turner, 2019). Des microorganismes (pas nécessairement les producteurs de PHA) sécrètent l'enzyme dans le milieu environnant (Fig. 12). La dPhaZ est composée d'un site de fixation qui lui permet de s'adsorber à la surface du PHA et d'un site catalytique qui hydrolyse le polymère en libérant dans le milieu des oligomères et monomères. De plus petites tailles (< 600 Da) (Jendrossek et al., 1996), les microorganismes peuvent les assimiler et les minéraliser pour compléter leur biodégradation (Fig. 12) (Numata et al., 2009).



**Figure 12 :** Cycle de biodégradation des PHA par les microorganismes en milieu naturel. Adapté de Numata et al., 2009

Les dPhaZ sont spécifiques à la longueur de la chaîne latérale carbonée du PHA, avec des enzymes distinctes impliquées dans la dépolymérisation des chaînes courtes (*scl*- ; 3 à 5 atomes de carbone) et d'autres dans la dépolymérisation des chaînes moyennes (*mcl*- ; 6 à 14 atomes de carbone) (Jendrossek and Handrick, 2002). Par exemple, dPhaZ<sub>SCL</sub> de *Pseudomonas lemoignei* n'a montré aucune activité sur les *mcl*-PHA. Inversement, une dPhaZ<sub>MCL</sub> de *Pseudomonas fluorescens* est inactive sur les *scl*-PHA (Jendrossek and Handrick, 2002). Les PHA dépolymérase dégradant les *scl*-PHA sont les plus étudiées car les *mcl*-PHA ne sont pas produits en quantités industrielles. Une base de données dédiée aux PHA dépolymérase a été créée (DED, <http://www.ded.uni-stuttgart.de> ; Knoll et al, 2009) pour répertorier toutes les PhaZ. En 2002, déjà 19 gènes codant pour des dPhaZ<sub>SCL</sub> avaient été clonés, analysés et répertoriés dans cette base de données et la découverte de nouvelles PHA synthases se poursuit encore plus récemment avec de nouveaux outils d'analyses génomiques (Bano et al., 2019; Jendrossek and Handrick, 2002; Morohoshi et al., 2020).

Les dPhaZ<sub>SCL</sub> sont composées de plusieurs domaines : le signal peptidique (SP, Fig. 13) qui permet à la protéine de traverser la membrane cytoplasmique, un domaine catalytique et une lipase box, un domaine de fixation (Substrate Binding Domain, SBD) lié au domaine catalytique par une région de liaison (linker) (Fig. 13). Il existe deux types de domaine catalytique en fonction de la disposition des acides aminés. Le type 1 est constitué d'un motif Histidine (H) – Sérine (S) – Aspartate (D) – Histidine tandis que l'histidine de départ du type 1 est placée en C terminale du domaine catalytique dans le type 2 (Sérine – Aspartate – Histidine – Histidine) (Fig. 13). Dans tous les cas, un site actif composé de trois acides aminés conservés (Sérine, Aspartate et histidine) est nécessaire à l'activité catalytique.



**Figure 13** : Types de structures des dPhaZ<sub>SCL</sub>. Structure avec un domaine de type 1 et de type 2 toutes deux composées de la triade catalytique (S – D – H). Adaptée de Jendrossek and Handrick, 2002.

Les dPhaZ<sub>SCL</sub> se fixent aux *scl*-PHA par l'intermédiaire du SDB. Le SDB constitue le domaine C-terminal de l'enzyme et regroupent entre 40 et 60 acides aminés (Jendrossek and Handrick, 2002). Ce domaine est responsable de la reconnaissance du substrat car il ne se lie pas à la cellulose ou la chitine (Kasuya et al., 1999). Deux types de SBD peuvent être différenciés (SBD1, SBD2, figure 13). Dans le type 1, une deuxième cystéine est présente. Des dPhaZ<sub>SCL</sub> ont également montré la présence de deux SBD, c'est le cas de la bactérie marine *Alcaligenes faecalis* AE122 (Kita et al., 1997). Les raisons de ces différences de structure sont peu discutées dans la littérature. Cependant, les acides aminés du SDB pourraient jouer un rôle dans la structure même de la protéine ou encore dans la liaison à la chaîne des *scl*-PHA par interactions hydrophobe et/electrostatique (Yamashita et al., 2001).

## Références

- Abe, H., Ishii, N., Sato, S., Tsuge, T., 2012. Thermal properties and crystallization behaviors of medium-chain-length poly(3-hydroxyalkanoate)s. *Polymer* 53, 3026–3034. <https://doi.org/10.1016/j.polymer.2012.04.043>
- Alauddin, M., Choudhury, I.A., El Baradie, M.A., Hashmi, M.S.J., 1995. Plastics and their machining: A review. *Journal of Materials Processing Technology* 54, 40–46. [https://doi.org/10.1016/0924-0136\(95\)01917-0](https://doi.org/10.1016/0924-0136(95)01917-0)
- Almstrand, R., Daims, H., Persson, F., Sörensson, F., Hermansson, M., 2013. New methods for analysis of spatial distribution and coaggregation of microbial populations in complex biofilms. *Appl Environ Microbiol* 79, 5978–5987. <https://doi.org/10.1128/AEM.01727-13>
- Amelia, T.S.M., Govindasamy, S., Tamothran, A.M., Vigneswari, S., Bhubalan, K., 2019. Applications of PHA in Agriculture, in: Kalia, V.C. (Ed.), *Biotechnological Applications of Polyhydroxyalkanoates*. Springer Singapore, Singapore, pp. 347–361. [https://doi.org/10.1007/978-981-13-3759-8\\_13](https://doi.org/10.1007/978-981-13-3759-8_13)
- Anjum, A., Zuber, M., Zia, K.M., Noreen, A., Anjum, M.N., Tabasum, S., 2016. Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: A review of recent advancements. *International Journal of Biological Macromolecules* 89, 161–174. <https://doi.org/10.1016/j.ijbiomac.2016.04.069>
- Auta, H.S., Emenike, C.U., Fauziah, S.H., 2017. Distribution and importance of microplastics in the marine environment: A review of the sources, fate, effects, and potential solutions. *Environment International* 102, 165–176. <https://doi.org/10.1016/j.envint.2017.02.013>
- Auta, H.S., Emenike, C.U., Jayanthi, B., Fauziah, S.H., 2018. Growth kinetics and biodeterioration of polypropylene microplastics by *Bacillus* sp. and *Rhodococcus* sp. isolated from mangrove sediment. *Marine Pollution Bulletin* 127, 15–21. <https://doi.org/10.1016/j.marpolbul.2017.11.036>
- Baekeland, L.H., 1909. The Synthesis, Constitution, and Uses of Bakelite. *J. Ind. Eng. Chem.* 1, 149–161. <https://doi.org/10.1021/ie50003a004>
- Baker, I., 2018. *Fifty Materials That Make the World*. Springer International Publishing, Cham. <https://doi.org/10.1007/978-3-319-78766-4>
- Bano, K., Kuddus, M., Zaheer, M.R., Zaheer, R.R., 2019. A novel, thermotolerant, extracellular PHB depolymerase producer *Paenibacillus alvei* PHB28 for bioremediation of biodegradable plastics. *Turkish Journal of Biochemistry* 44, 344–353. <https://doi.org/10.1515/tjb-2018-0207>
- Barnes, D.K.A., 2002. Biodiversity: invasions by marine life on plastic debris. *Nature* 416, 808–809. <https://doi.org/10.1038/416808a>
- Basili, M., Quero, G.M., Giovannelli, D., Manini, E., Vignaroli, C., Avio, C.G., De Marco, R., Luna, G.M., 2020. Major Role of Surrounding Environment in Shaping Biofilm Community Composition on Marine Plastic Debris. *Frontiers in Marine Science* 7.
- Beckman, E., 2018. Vous reprendrez bien un peu de plastique ? The Conversation. URL <http://theconversation.com/vous-reprendrez-bien-un-peu-de-plastique-101579> (accessed 3.2.23).
- Brandi, H., Bachofen, R., Mayer, J., Wintermantel, E., 1995. Degradation and applications of polyhydroxyalkanoates. *Can. J. Microbiol.* 41, 143–153. <https://doi.org/10.1139/m95-181>
- Brandl, H., Knee, E.J., Fuller, R.C., Gross, R.A., Lenz, R.W., 1989. Ability of the phototrophic bacterium *Rhodospirillum rubrum* to produce various poly ( $\beta$ -hydroxyalkanoates):



- Potential sources for biodegradable polyesters. *International Journal of Biological Macromolecules* 11, 49–55. [https://doi.org/10.1016/0141-8130\(89\)90040-8](https://doi.org/10.1016/0141-8130(89)90040-8)
- Browne, M.A., Galloway, T., Thompson, R., 2007. Microplastic an emerging contaminant of potential concern ? *Integr Environ Assess Manag* 3, 559–561. <https://doi.org/10.1002/ieam.5630030412>
- Bruzaud, S., 2021. Polyesters biosourcés et/ou biodégradables - De l'élaboration à la fin de vie. *Chimie verte*. <https://doi.org/10.51257/a-v1-chv4039>
- Bryant, J.A., Clemente, T.M., Viviani, D.A., Fong, A.A., Thomas, K.A., Kemp, P., Karl, D.M., White, A.E., DeLong, E.F., 2016. Diversity and Activity of Communities Inhabiting Plastic Debris in the North Pacific Gyre. *mSystems* 1, 10.1128/msystems.00024-16. <https://doi.org/10.1128/msystems.00024-16>
- Bugnicourt, E., Cinelli, P., Lazzeri, A., Alvarez, V.A., 2014. Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging. <https://doi.org/10.3144/expresspolymlett.2014.82>
- Castro-Aguirre, E., Auras, R., Selke, S., Rubino, M., Marsh, T., 2018. Enhancing the biodegradation rate of poly(lactic acid) films and PLA bio-nanocomposites in simulated composting through bioaugmentation. *Polymer Degradation and Stability* 154, 46–54. <https://doi.org/10.1016/j.polymdegradstab.2018.05.017>
- Chalmin, P., 2019. Comment le monde s'est plastifié. *The Conversation*. URL <http://theconversation.com/comment-le-monde-s'est-plastifie-115991> (accessed 3.2.23).
- Ciesielski, S., Możejko, J., Pisutpaisal, N., 2015. Plant oils as promising substrates for polyhydroxyalkanoates production. *Journal of Cleaner Production, Bridges for a more sustainable future: Joining Environmental Management for Sustainable Universities (EMSU) and the European Roundtable for Sustainable Consumption and Production (ERSCP) conferences* 106, 408–421. <https://doi.org/10.1016/j.jclepro.2014.09.040>
- Conan, P., Philip, L., Ortega-Retuerta, E., Odobel, C., Duran, C., Pandin, C., Giraud, C., Meistertzheim, A.-L., Barbe, V., Ter Hall, A., Pujo-Pay, M., Ghiglione, J.-F., 2022. Evidence of coupled autotrophy and heterotrophy on plastic biofilms and its influence on surrounding seawater. *Environmental Pollution* 315, 120463. <https://doi.org/10.1016/j.envpol.2022.120463>
- Corre, Y.-M., Bruzaud, S., Audic, J.-L., Grohens, Y., 2012. Morphology and functional properties of commercial polyhydroxyalkanoates: A comprehensive and comparative study. *Polymer Testing* 31, 226–235. <https://doi.org/10.1016/j.polymertesting.2011.11.002>
- Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., Marrie, T.J., 1987. Bacterial biofilms in nature and disease. *Annu Rev Microbiol* 41, 435–464. <https://doi.org/10.1146/annurev.mi.41.100187.002251>
- De Tender, C., Devriese, L.I., Haegeman, A., Maes, S., Vangeyte, J., Cattrijsse, A., Dawyndt, P., Ruttink, T., 2017. Temporal Dynamics of Bacterial and Fungal Colonization on Plastic Debris in the North Sea. *Environ Sci Technol* 51, 7350–7360. <https://doi.org/10.1021/acs.est.7b00697>
- Debroas, D., Mone, A., Ter Halle, A., 2017. Plastics in the North Atlantic garbage patch: A boat-microbe for hitchhikers and plastic degraders. *Science of The Total Environment* 599–600, 1222–1232. <https://doi.org/10.1016/j.scitotenv.2017.05.059>
- Deroiné, M., César, G., Le Duigou, A., Davies, P., Bruzaud, S., 2015. Natural Degradation and Biodegradation of Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) in Liquid and Solid Marine Environments. *J Polym Environ* 23, 493–505. <https://doi.org/10.1007/s10924-015-0736-5>

- Deroiné, M., Le Duigou, A., Corre, Y.-M., Le Gac, P.-Y., Davies, P., César, G., Bruzard, S., 2014. Seawater accelerated ageing of poly(3-hydroxybutyrate-co-3-hydroxyvalerate). *Polymer Degradation and Stability* 105, 237–247. <https://doi.org/10.1016/j.polymdegradstab.2014.04.026>
- Doi, Y., Kanesawa, Y., Kunioka, M., Saito, T., 1990. Biodegradation of microbial copolyesters: poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and poly(3-hydroxybutyrate-co-4-hydroxybutyrate). *Macromolecules* 23, 26–31. <https://doi.org/10.1021/ma00203a006>
- Donlan, R.M., 2002. Biofilms: Microbial Life on Surfaces. *Emerg Infect Dis* 8, 881–890. <https://doi.org/10.3201/eid0809.020063>
- Dussud, Claire, Hudec, C., George, M., Fabre, P., Higgs, P., Bruzard, S., Delort, A.-M., Eyheraguibel, B., Meistertzheim, A.-L., Jacquin, J., Cheng, J., Callac, N., Odobel, C., Rabouille, S., Ghiglione, J.-F., 2018. Colonization of Non-biodegradable and Biodegradable Plastics by Marine Microorganisms. *Frontiers in Microbiology* 9. <https://doi.org/10.3389/fmicb.2018.01571>
- Dussud, C., Meistertzheim, A.L., Conan, P., Pujó-Pay, M., George, M., Fabre, P., Coudane, J., Higgs, P., Elineau, A., Pedrotti, M.L., Gorsky, G., Ghiglione, J.F., 2018. Evidence of niche partitioning among bacteria living on plastics, organic particles and surrounding seawaters. *Environ Pollut* 236, 807–816. <https://doi.org/10.1016/j.envpol.2017.12.027>
- Elifantz, H., Horn, G., Ayon, M., Cohen, Y., Minz, D., 2013. Rhodobacteraceae are the key members of the microbial community of the initial biofilm formed in Eastern Mediterranean coastal seawater. *FEMS Microbiology Ecology* 85, 348–357. <https://doi.org/10.1111/1574-6941.12122>
- European Bioplastics, nova-institute, 2022. European Bioplastics. European Bioplastics e.V. URL <https://www.european-bioplastics.org/bioplastics/materials/> (accessed 7.7.23).
- Farah, S., Anderson, D.G., Langer, R., 2016. Physical and mechanical properties of PLA, and their functions in widespread applications — A comprehensive review. *Advanced Drug Delivery Reviews, PLA biodegradable polymers* 107, 367–392. <https://doi.org/10.1016/j.addr.2016.06.012>
- Flemming, H.-C., Wingender, J., 2010. The biofilm matrix. *Nat Rev Microbiol* 8, 623–633. <https://doi.org/10.1038/nrmicro2415>
- Flemming, H.-C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A., Kjelleberg, S., 2016. Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol* 14, 563–575. <https://doi.org/10.1038/nrmicro.2016.94>
- Fontanella, S., Bonhomme, S., Koutny, M., Husarova, L., Brusson, J.-M., Courdavault, J.-P., Pitteri, S., Samuel, G., Pichon, G., Lemaire, J., Delort, A.-M., 2010. Comparison of the biodegradability of various polyethylene films containing pro-oxidant additives. *Polymer Degradation and Stability* 95, 1011–1021. <https://doi.org/10.1016/j.polymdegradstab.2010.03.009>
- Fukui, T., Doi, Y., 1997. Cloning and analysis of the poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) biosynthesis genes of *Aeromonas caviae*. *J Bacteriol* 179, 4821–4830.
- Galli, P., 1994. The breakthrough in catalysis and processes for olefin polymerization: Innovative structures and a strategy in the materials area for the twenty-first century. *Progress in Polymer Science* 19, 959–974. [https://doi.org/10.1016/0079-6700\(94\)90016-7](https://doi.org/10.1016/0079-6700(94)90016-7)
- GESAMP, 2015. Sources, Fate and Effects of Microplastics in the Marine Environment: A Global Assessment. p98.
- Geyer, R., 2020. A Brief History of Plastics, Streit-Bianchi, M., Cimadevila, M., Trettnak, W. (Eds.), *Mare Plasticum - The Plastic Sea: Combatting Plastic Pollution Through Science*

- and Art. Springer International Publishing, Cham, pp. 31–47. [https://doi.org/10.1007/978-3-030-38945-1\\_2](https://doi.org/10.1007/978-3-030-38945-1_2)
- Ghiglione, J.F., Dussud, C., 2014. La dégradation des plastiques en mer. *sfecologie.org*. URL <https://www.sfecologie.org/regard/r63-plastiques-en-mer-dussud-et-ghiglione/> (accessed 5.31.21).
- Goh, L.-K., Purama, R.K., Sudesh, K., 2014. Enhancement of Stress Tolerance in the Polyhydroxyalkanoate Producers without Mobilization of the Accumulated Granules. *Appl Biochem Biotechnol* 172, 1585–1598. <https://doi.org/10.1007/s12010-013-0634-z>
- Grage, K., Jahns, A.C., Parlane, N., Palanisamy, R., Rasiah, I.A., Atwood, J.A., Rehm, B.H.A., 2009. Bacterial Polyhydroxyalkanoate Granules: Biogenesis, Structure, and Potential Use as Nano-/Micro-Beads in Biotechnological and Biomedical Applications. *Biomacromolecules* 10, 660–669. <https://doi.org/10.1021/bm801394s>
- Harrison, J., Sapp, M., Schratzberger, M., Osborn, A., 2011. Interactions Between Microorganisms and Marine Microplastics: A Call for Research. *Marine Technology Society Journal* 45, 12–20. <https://doi.org/10.4031/MTSJ.45.2.2>
- Harrison, J.P., Schratzberger, M., Sapp, M., Osborn, A.M., 2014. Rapid bacterial colonization of low-density polyethylene microplastics in coastal sediment microcosms. *BMC Microbiology* 14, 232. <https://doi.org/10.1186/s12866-014-0232-4>
- Ho, B.T., Roberts, T.K., Lucas, S., 2018. An overview on biodegradation of polystyrene and modified polystyrene: the microbial approach. *Critical Reviews in Biotechnology* 38, 308–320. <https://doi.org/10.1080/07388551.2017.1355293>
- Hugenholtz, P., Hooper, S.D., Kyrpides, N.C., 2009. Focus: Synergistetes. *Environ Microbiol* 11, 1327–1329. <https://doi.org/10.1111/j.1462-2920.2009.01949.x>
- Jacquín, J., Cheng, J., Odobel, C., Pandin, C., Conan, P., Pujó-Pay, M., Barbe, V., Meistertzheim, A.-L., Ghiglione, J.-F., 2019. Microbial Ecotoxicology of Marine Plastic Debris: A Review on Colonization and Biodegradation by the “Plastisphere.” *Front. Microbiol.* 10. <https://doi.org/10.3389/fmicb.2019.00865>
- Jendrossek, D., Handrick, R., 2002. Microbial Degradation of Polyhydroxyalkanoates. *Annu. Rev. Microbiol.* 56, 403–432. <https://doi.org/10.1146/annurev.micro.56.012302.160838>
- Jendrossek, D., Schirmer, A., Schlegel, H.G., 1996. Biodegradation of polyhydroxyalkanoic acids. *Applied Microbiology and Biotechnology* 46, 451–463. <https://doi.org/10.1007/s002530050844>
- Kadouri, D., Jurkevitch, E., Okon, Y., Castro-Sowinski, S., 2005. Ecological and Agricultural Significance of Bacterial Polyhydroxyalkanoates. *Critical Reviews in Microbiology* 31, 55–67. <https://doi.org/10.1080/10408410590899228>
- Kasuya, K., Ohura, T., Masuda, K., Doi, Y., 1999. Substrate and binding specificities of bacterial polyhydroxybutyrate depolymerases. *Int J Biol Macromol* 24, 329–336. [https://doi.org/10.1016/s0141-8130\(99\)00046-x](https://doi.org/10.1016/s0141-8130(99)00046-x)
- Kirstein, I.V., Wichels, A., Krohne, G., Gerdts, G., 2018. Mature biofilm communities on synthetic polymers in seawater - Specific or general? *Marine Environmental Research* 142, 147–154. <https://doi.org/10.1016/j.marenvres.2018.09.028>
- Kita, K., Mashiba, S., Nagita, M., Ishimaru, K., Okamoto, K., Yanase, H., Kato, N., 1997. Cloning of poly(3-hydroxybutyrate) depolymerase from a marine bacterium, *Alcaligenes faecalis* AE122, and characterization of its gene product. *Biochim Biophys Acta* 1352, 113–122. [https://doi.org/10.1016/s0167-4781\(97\)00011-0](https://doi.org/10.1016/s0167-4781(97)00011-0)
- Knoll, M., Hamm, T.M., Wagner, F., Martinez, V., Pleiss, J., 2009. The PHA Depolymerase Engineering Database: A systematic analysis tool for the diverse family of

- polyhydroxyalkanoate (PHA) depolymerases. *BMC Bioinformatics* 10, 89. <https://doi.org/10.1186/1471-2105-10-89>
- Koller, M., Mukherjee, A., 2022. A New Wave of Industrialization of PHA Biopolyesters. *Bioengineering* 9, 74. <https://doi.org/10.3390/bioengineering9020074>
- Koller, M., Salerno, A., Dias, M.M. de S., Reiterer, A., Braunegg, G., 2010. Modern Biotechnological Polymer Synthesis: A Review. *Food technology and biotechnology* 48, 255–269.
- Lear, G., Kingsbury, J.M., Franchini, S., Gambarini, V., Maday, S.D.M., Wallbank, J.A., Weaver, L., Pantos, O., 2021. Plastics and the microbiome: impacts and solutions. *Environmental Microbiome* 16, 2. <https://doi.org/10.1186/s40793-020-00371-w>
- Lebreton, L.C.-M., Greer, S.D., Borrero, J.C., 2012. Numerical modelling of floating debris in the world's oceans. *Marine Pollution Bulletin* 64, 653–661. <https://doi.org/10.1016/j.marpolbul.2011.10.027>
- Lee, J.-W., Nam, J.-H., Kim, Y.-H., Lee, K.-H., Lee, D.-H., 2008. Bacterial communities in the initial stage of marine biofilm formation on artificial surfaces. *J Microbiol.* 46, 174–182. <https://doi.org/10.1007/s12275-008-0032-3>
- Lemechko, P., Le Fellic, M., Bruzard, S., 2019. Production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) using agro-industrial effluents with tunable proportion of 3-hydroxyvalerate monomer units. *International Journal of Biological Macromolecules* 128, 429–434. <https://doi.org/10.1016/j.ijbiomac.2019.01.170>
- Liebergessell, M., Steinbüchel, A., 1992. Cloning and nucleotide sequences of genes relevant for biosynthesis of poly(3-hydroxybutyric acid) in *Chromatium vinosum* strain D. *Eur J Biochem* 209, 135–150. <https://doi.org/10.1111/j.1432-1033.1992.tb17270.x>
- Luengo, J.M., García, B., Sandoval, A., Naharro, G., Olivera, E.R., 2003. Bioplastics from microorganisms. *Current Opinion in Microbiology* 6, 251–260. [https://doi.org/10.1016/S1369-5274\(03\)00040-7](https://doi.org/10.1016/S1369-5274(03)00040-7)
- Manger, C., 2022. European Bioplastic, Facts and Figures, 2021. *European Bioplastics e.V.* URL <https://www.european-bioplastics.org/news/publications/> (accessed 3.10.23).
- Maunder, E., Welch, M., 2017. Matrix exopolysaccharides; the sticky side of biofilm formation. *FEMS Microbiol Lett* 364, fnx120. <https://doi.org/10.1093/femsle/fnx120>
- Morales, S.E., Holben, W.E., 2011. Linking bacterial identities and ecosystem processes: can 'omic' analyses be more than the sum of their parts? *FEMS Microbiology Ecology* 75, 2–16. <https://doi.org/10.1111/j.1574-6941.2010.00938.x>
- Morohoshi, T., Oi, T., Suzuki, T., Sato, S., 2020. Identification and characterization of a novel extracellular polyhydroxyalkanoate depolymerase in the complete genome sequence of *Undibacterium* sp. KW1 and YM2 strains. *PLOS ONE* 15, e0232698. <https://doi.org/10.1371/journal.pone.0232698>
- Moura, V., Ribeiro, I., Moriggi, P., Capão, A., Salles, C., Bitati, S., Procópio, L., 2018. The influence of surface microbial diversity and succession on microbiologically influenced corrosion of steel in a simulated marine environment. *Arch Microbiol* 200, 1447–1456. <https://doi.org/10.1007/s00203-018-1559-2>
- Numata, K., Abe, H., Iwata, T., 2009. Biodegradability of Poly(hydroxyalkanoate) Materials. *Materials* 2, 1104–1126. <https://doi.org/10.3390/ma2031104>
- Oberbeckmann, S., Kreikemeyer, B., Labrenz, M., 2017. Environmental Factors Support the Formation of Specific Bacterial Assemblages on Microplastics. *Front Microbiol* 8, 2709. <https://doi.org/10.3389/fmicb.2017.02709>
- Oberbeckmann, S., Labrenz, M., 2020. Marine Microbial Assemblages on Microplastics: Diversity, Adaptation, and Role in Degradation. *Ann Rev Mar Sci* 12, 209–232. <https://doi.org/10.1146/annurev-marine-010419-010633>

- Oberbeckmann, S., Osborn, A.M., Duhaime, M.B., 2016. Microbes on a Bottle: Substrate, Season and Geography Influence Community Composition of Microbes Colonizing Marine Plastic Debris. *PLOS ONE* 11, e0159289. <https://doi.org/10.1371/journal.pone.0159289>
- Obruca, S., Sedlacek, P., Slaninova, E., Fritz, I., Daffert, C., Meixner, K., Sedrlova, Z., Koller, M., 2020. Novel unexpected functions of PHA granules. *Appl Microbiol Biotechnol* 104, 4795–4810. <https://doi.org/10.1007/s00253-020-10568-1>
- Odobel, C., Dussud, C., Philip, L., Derippe, G., Lauters, M., Eyheraguibel, B., Burgaud, G., Ter Halle, A., Meistertzheim, A.-L., Bruzard, S., Barbe, V., Ghiglione, J.-F., 2021. Bacterial Abundance, Diversity and Activity During Long-Term Colonization of Non-biodegradable and Biodegradable Plastics in Seawater. *Frontiers in Microbiology* 12. <https://doi.org/10.3389/fmicb.2021.734782>
- O'Toole, G.A., Wong, G.C., 2016. Sensational biofilms: surface sensing in bacteria. *Curr Opin Microbiol* 30, 139–146. <https://doi.org/10.1016/j.mib.2016.02.004>
- Pachekoski, W.M., Agnelli, J.A.M., Belem, L.P., 2009. Thermal, mechanical and morphological properties of poly (hydroxybutyrate) and polypropylene blends after processing. *Materials Research* 12, 159–164. <https://doi.org/10.1590/S1516-14392009000200008>
- Peñas, M.I., Pérez-Camargo, R.A., Hernández, R., Müller, A.J., 2022. A Review on Current Strategies for the Modulation of Thermomechanical, Barrier, and Biodegradation Properties of Poly (Butylene Succinate) (PBS) and Its Random Copolymers. *Polymers* 14, 1025. <https://doi.org/10.3390/polym14051025>
- Pérez-Rivero, C., Hernandez-Raquet, G., 2017. Polyhydroxyalcanoates : une alternative ‘bio’ aux plastiques traditionnels. *Innovations Agronomiques* 58, 99–112.
- Philip, S., Keshavarz, T., Roy, I., 2007. Polyhydroxyalkanoates: biodegradable polymers with a range of applications. *Journal of Chemical Technology & Biotechnology* 82, 233–247. <https://doi.org/10.1002/jctb.1667>
- Pinnell, L.J., Turner, J.W., 2019. Shotgun Metagenomics Reveals the Benthic Microbial Community Response to Plastic and Bioplastic in a Coastal Marine Environment. *Frontiers in Microbiology* 10.
- Plastic Europe, 2023. Plastics - The facts 2022. Plastics Europe FR. URL <https://plasticseurope.org/fr/knowledge-hub/plastics-the-facts-2022/> (accessed 7.28.23).
- Plastic Europe, 2021. Plastics -The Facts 2020. URL <https://plasticseurope.org/knowledge-hub/plastics-the-facts-2020/> (accessed 3.7.23).
- Pompilio, A., Piccolomini, R., Picciani, C., D'Antonio, D., Savini, V., Di Bonaventura, G., 2008. Factors associated with adherence to and biofilm formation on polystyrene by *Stenotrophomonas maltophilia*: the role of cell surface hydrophobicity and motility. *FEMS Microbiology Letters* 287, 41–47. <https://doi.org/10.1111/j.1574-6968.2008.01292.x>
- Poretsky, R., Rodriguez-R, L.M., Luo, C., Tsementzi, D., Konstantinidis, K.T., 2014. Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics. *PLoS One* 9, e93827. <https://doi.org/10.1371/journal.pone.0093827>
- Prieto, A., Escapa, I.F., Martínez, V., Dinjaski, N., Herencias, C., de la Peña, F., Tarazona, N., Revelles, O., 2016. A holistic view of polyhydroxyalkanoate metabolism in *Pseudomonas putida*. *Environ. Microbiol.* 18, 341–357. <https://doi.org/10.1111/1462-2920.12760>
- Rehm, B.H.A., 2003. Polyester synthases: natural catalysts for plastics. *Biochemical Journal* 376, 15–33. <https://doi.org/10.1042/bj20031254>

- Rochman, C.M., 2015. The Complex Mixture, Fate and Toxicity of Chemicals Associated with Plastic Debris in the Marine Environment, in: Bergmann, M., Gutow, L., Klages, M. (Eds.), *Marine Anthropogenic Litter*. Springer International Publishing, Cham, pp. 117–140. [https://doi.org/10.1007/978-3-319-16510-3\\_5](https://doi.org/10.1007/978-3-319-16510-3_5)
- Saeki, Y., Emura, T., 2002. Technical progresses for PVC production. *Progress in Polymer Science* 27, 2055–2131. [https://doi.org/10.1016/S0079-6700\(02\)00039-4](https://doi.org/10.1016/S0079-6700(02)00039-4)
- Sashiwa, H., Fukuda, R., Okura, T., Sato, S., Nakayama, A., 2018. Microbial Degradation Behavior in Seawater of Polyester Blends Containing Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx). *Marine Drugs* 16, 34. <https://doi.org/10.3390/md16010034>
- Shah, A.A., Hasan, F., Hameed, A., Ahmed, S., 2008. Biological degradation of plastics: A comprehensive review. *Biotechnology Advances* 26, 246–265. <https://doi.org/10.1016/j.biotechadv.2007.12.005>
- Silva, J.B., Pereira, J.R., Marreiros, B.C., Reis, M.A.M., Freitas, F., 2021. Microbial production of medium-chain length polyhydroxyalkanoates. *Process Biochemistry* 102, 393–407. <https://doi.org/10.1016/j.procbio.2021.01.020>
- Singh, A.K., Sharma, L., Srivastava, J.K., Mallick, N., Ansari, M.I., 2018. Microbially Originated Polyhydroxyalkanoate (PHA) Biopolymers: An Insight into the Molecular Mechanism and Biogenesis of PHA Granules, in: Singh, O.V., Chandel, A.K. (Eds.), *Sustainable Biotechnology- Enzymatic Resources of Renewable Energy*. Springer International Publishing, Cham, pp. 355–398. [https://doi.org/10.1007/978-3-319-95480-6\\_14](https://doi.org/10.1007/978-3-319-95480-6_14)
- Sørensen, S.J., Bailey, M., Hansen, L.H., Kroer, N., Wuertz, S., 2005. Studying plasmid horizontal transfer in situ: a critical review. *Nat Rev Microbiol* 3, 700–710. <https://doi.org/10.1038/nrmicro1232>
- Soto, G., Setten, L., Lisi, C., Maurelis, C., Mozzicafreddo, M., Cuccioloni, M., Angeletti, M., Ayub, N.D., 2012. Hydroxybutyrate prevents protein aggregation in the halotolerant bacterium *Pseudomonas* sp. CT13 under abiotic stress. *Extremophiles* 16, 455–462. <https://doi.org/10.1007/s00792-012-0445-0>
- Sudesh, K., Abe, H., Doi, Y., 2000. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Progress in Polymer Science* 25, 1503–1555. [https://doi.org/10.1016/S0079-6700\(00\)00035-6](https://doi.org/10.1016/S0079-6700(00)00035-6)
- Sun, Z., Ramsay, J., Guay, M., Ramsay, B., 2009. Enhanced yield of medium-chain-length polyhydroxyalkanoates from nonanoic acid by co-feeding glucose in carbon-limited, fed-batch culture. *Journal of Biotechnology* 143, 262–267. <https://doi.org/10.1016/j.jbiotec.2009.07.014>
- Sznajder, A., Jendrossek, D., 2011. Biochemical characterization of a new type of intracellular PHB depolymerase from *Rhodospirillum rubrum* with high hydrolytic activity on native PHB granules. *Appl Microbiol Biotechnol* 89, 1487–1495. <https://doi.org/10.1007/s00253-011-3096-7>
- Titow, W.V., 2012. *PVC Plastics: Properties, Processing, and Applications*. Springer Science & Business Media.
- Trivedi, A.K., Gupta, M.K., Singh, H., 2023. PLA based biocomposites for sustainable products: A review. *Advanced Industrial and Engineering Polymer Research*. <https://doi.org/10.1016/j.aiepr.2023.02.002>
- Vimala, P.P., Mathew, L., 2016. Biodegradation of Polyethylene Using *Bacillus Subtilis*. *Procedia Technology, International Conference on Emerging Trends in Engineering, Science and Technology (ICETEST - 2015)* 24, 232–239. <https://doi.org/10.1016/j.protcy.2016.05.031>

- Volant, C., Balnois, E., Vignaud, G., Magueresse, A., Bruzard, S., 2022. Design of Polyhydroxyalkanoate (PHA) Microbeads with Tunable Functional Properties and High Biodegradability in Seawater. *J Polym Environ* 30, 2254–2269. <https://doi.org/10.1007/s10924-021-02345-6>
- Wu, D., He, J., Gong, Y., Chen, D., Zhu, X., Qiu, N., Sun, M., Li, M., Yu, Z., 2011. Proteomic analysis reveals the strategies of *Bacillus thuringiensis* YBT-1520 for survival under long-term heat stress. *Proteomics* 11, 2580–2591. <https://doi.org/10.1002/pmic.201000392>
- Xie, T., McAuley, K.B., Hsu, J.C.C., Bacon, D.W., 1994. Gas Phase Ethylene Polymerization: Production Processes, Polymer Properties, and Reactor Modeling. *Ind. Eng. Chem. Res.* 33, 449–479. <https://doi.org/10.1021/ie00027a001>
- Yamashita, K., Aoyagi, Y., Abe, H., Doi, Y., 2001. Analysis of Adsorption Function of Polyhydroxybutyrate Depolymerase from *Alcaligenes faecalis* T1 by Using a Quartz Crystal Microbalance. *Biomacromolecules* 2, 25–28. <https://doi.org/10.1021/bm0000844>
- Yang, J., Yang, Y., Wu, W.-M., Zhao, J., Jiang, L., 2014. Evidence of Polyethylene Biodegradation by Bacterial Strains from the Guts of Plastic-Eating Waxworms. *Environ. Sci. Technol.* 48, 13776–13784. <https://doi.org/10.1021/es504038a>
- Yin, J., Chen, J.-C., Wu, Q., Chen, G.-Q., 2015. Halophiles, coming stars for industrial biotechnology. *Biotechnol Adv* 33, 1433–1442. <https://doi.org/10.1016/j.biotechadv.2014.10.008>
- Zalasiewicz, J., Waters, C.N., Ivar do Sul, J.A., Corcoran, P.L., Barnosky, A.D., Cearreta, A., Edgeworth, M., Gałuszka, A., Jeandel, C., Leinfelder, R., McNeill, J.R., Steffen, W., Summerhayes, C., Wapreisch, M., Williams, M., Wolfe, A.P., Yonan, Y., 2016. The geological cycle of plastics and their use as a stratigraphic indicator of the Anthropocene. *Anthropocene* 13, 4–17. <https://doi.org/10.1016/j.ancene.2016.01.002>
- Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A., 2013. Life in the “Plastisphere”: Microbial Communities on Plastic Marine Debris. *Environ. Sci. Technol.* 47, 7137–7146. <https://doi.org/10.1021/es401288x>
- Zhang, J., Shishatskaya, E.I., Volova, T.G., da Silva, L.F., Chen, G.-Q., 2018. Polyhydroxyalkanoates (PHA) for therapeutic applications. *Materials Science and Engineering: C* 86, 144–150. <https://doi.org/10.1016/j.msec.2017.12.035>
- Zinn, M., 2010. Biosynthesis of Medium-Chain-Length Poly[(R)-3-hydroxyalkanoates], in: Chen, G.G.-Q. (Ed.), *Plastics from Bacteria: Natural Functions and Applications*, Microbiology Monographs. Springer, Berlin, Heidelberg, pp. 213–236. [https://doi.org/10.1007/978-3-642-03287-5\\_9](https://doi.org/10.1007/978-3-642-03287-5_9)

# Chapitre 2 : Biodégradation de plusieurs polyhydroxyalcanoates (PHA) produits à façon et étude des communautés bactériennes marines associées



## Préambule

Ce premier chapitre rédigé sous forme d'article scientifique en anglais, a été soumis à la revue *Journal of Hazardous Materials*.

Des études antérieures avaient montré les grandes capacités des communautés microbiennes à biodégrader les *scl*-PHA en milieu marin (Deroiné et al., 2014 ; Volant et al., 2022). Ces travaux ne prenaient pas en compte l'étude de la diversité microbienne associée, et d'autres travaux se sont intéressés à la diversité des microorganismes qui colonisent des PHA commerciaux (PHBHV) en conditions marines, sans faire de lien direct avec la biodégradation (Dussud et al., 2018 ; Odobel et al., 2021). D'autre part, aucune étude n'a jamais rapporté d'activité et de diversité microbienne sur les *mcl*-PHA, en raison de leurs faibles disponibilités commerciales. Malgré la grande variété de composition chimiques (et donc de propriétés), les PHA sont généralement considérés comme "biodégradables" alors qu'aucune étude n'a proposé de comparaison directe de la biodégradabilité des deux grandes classes de *scl*- et *mcl*-PHA.

Les objectifs de cette étude sont de (1) produire une palette de PHA appartenant aux *scl*- et *mcl*-PHA aux propriétés physico-chimiques variées, (2) analyser les signaux de biodégradation associés aux PHA produits en milieu marin et (3) étudier les communautés bactériennes présentes sur ces PHA et les principaux taxa impliqués dans leur biodégradation.

La méthode et les résultats présentés proviennent d'une collaboration multidisciplinaire entre polyméristes (laboratoires IRDL, IRMA et ICCF) et microbiologistes (laboratoires LOMIC, Genoscope et Plastic@Sea). Un total de 6 PHA (3 *scl*- et 3 *mcl*-PHA) ont été produits par fermentation bactérienne à partir de différentes sources de carbone et différentes souches bactériennes puis caractérisés. Les PHA ont par la suite subi une expérience de biodégradation de deux mois à partir de communautés bactériennes collectées sur du *scl*- ou *mcl*-PHA précédemment immergés pendant un mois dans la mer méditerranée. La biodégradation a été déterminée par suivi de la consommation en oxygène et suivi de l'activité hétérotrophe bactérienne. Les communautés bactériennes ont été identifiées par séquençage haut débit du gène codant pour l'ARNr 16S.

Les résultats montrent de manière originale que la structure chimique du polymère (c'est-à-dire la taille de la chaîne latérale pour les PHA à chaînes courtes ou moyennes) ainsi que la spécificité du biofilm se développant sur les films plastiques (c'est-à-dire la «plastisphère» associée) constituent les principaux moteurs de la biodégradabilité des PHA en milieu marin. Nous avons également montré que les caractéristiques physiques du PHA (cristallinité, transition vitreuse, hydrophobicité, poids moléculaire) étaient des facteurs

beaucoup moins importants dans les processus de biodégradabilité à cette échelle. Enfin, les communautés microbiennes des *mcl*-PHA ont présenté une plus grande diversité. *Marinobacter sp.* et *Cobetia sp.* étaient majoritaires sur les *scl*-PHA et contribuer fortement à expliquer la structure de la communautés des *scl*-PHA comparée aux *mcl*-PHA tandis que *Pseudomonas sp.* était majoritairement présentes sur les *mcl*-PHA

# Marine biodegradation of tailor-made polyhydroxyalkanoates (PHA) influenced by the chemical structure and associated bacterial communities

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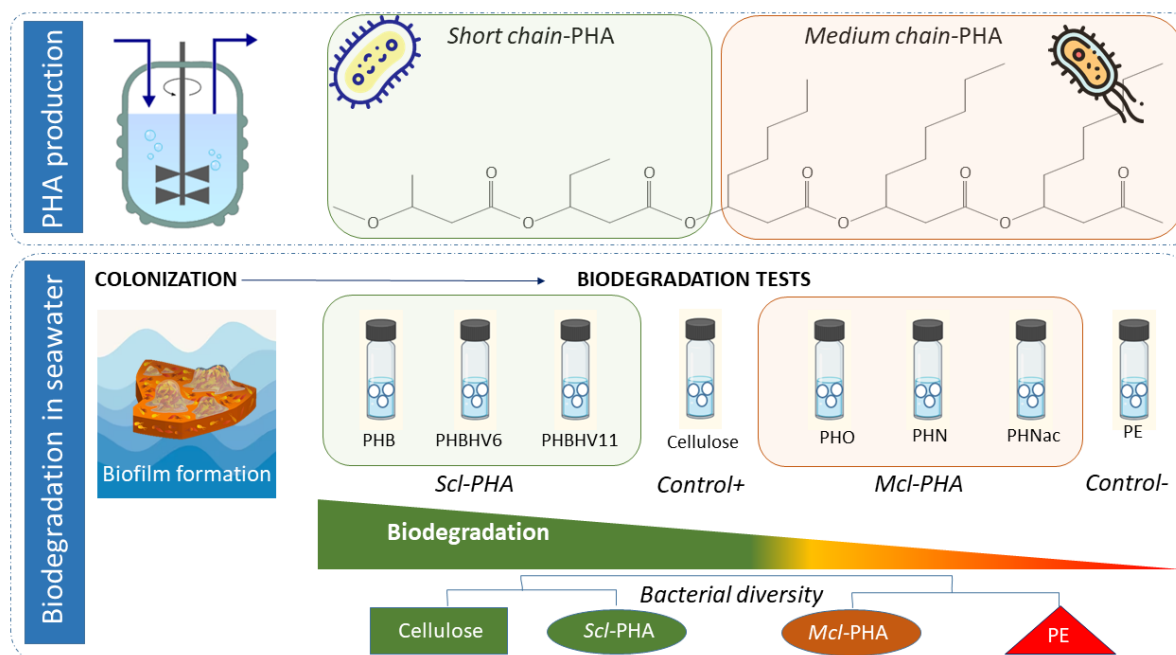
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**Keywords:** Polyhydroxyalkanoates (PHA), Biosynthesis, Biodegradation, Plastisphere

## Highlights

- Marine biodegradation is much higher on short chain-length (*scl*-) than on medium chain-length (*mcl*-) PHA.
- Physico-chemical properties (crystallinity, hydrophobicity, molecular weight) do not influence biodegradation at such scale.
- Distinct bacteria living on *scl*- vs *mcl*-PHA are playing a potential key role in biodegradation.

## Graphical abstract



## Abstract

In the recent years, biodegradable polymers have been proposed to reduce environmental impacts of plastics for specific applications. Production of polyhydroxyalkanoates (PHA) by using diverse carbon sources provides further benefits for the sustainable development of biodegradable plastics. Here, we present the first study evaluating the impact of physical, chemical and biological factors driving the biodegradability of various tailor-made PHA in the marine environment. Our multidisciplinary approach demonstrated that the chemical structure of the polymer (i.e. the side chain size for *short-* vs. *medium-chain* PHA) together with the specificity of the biofilm growing on plastic films (i.e. the associated ‘plastisphere’) were the main drivers of the PHA biodegradability in the marine environment. We also showed that the physical characteristics of the PHA (crystallinity, glass transition, hydrophobicity, molecular weight) were much less important factors influencing the biodegradability processes for the PHA family.

### 1. Introduction

Plastic pollution is nowadays a global and conscious crisis that is of major concern to marine organisms (Deudero and Alomar, 2015), but also contributes to raise global greenhouse

gas emissions and climate change (Shen et al., 2020). Marine environment constitutes a large reservoir of mismanaged plastic waste, with 4.8 to 12.7 million metric tons of plastic entering the oceans every year (Jambeck et al., 2015). As a partial solution, it has been proposed to manufacture plastics that would be both bio-based, i.e. made from renewable resources, and biodegradable (totally transformed in biomass and CO<sub>2</sub>) in a given environment (compost, soil, water) over a reasonable amount of time (weeks, months). Relevance of the use of biodegradable plastics has been listed for selected applications with respect to their use and end of life (Paul-Pont et al., 2023). Among the bio-sourced and biodegradable polymers, polyhydroxyalkanoates (PHA) are considered as a promising alternative to petroleum-based or non-biodegradable polymers. Mainly but not only from bacterial origin, the PHA constitute a large family and display a wide range of chemical compositions and properties according to the producing strain, the source of carbon used for feeding and the fermentation process. PHA can be divided into two subgroups: short chain-length PHA (*scl*-PHA) composed of 3 to 5 carbon atoms and medium chain-length PHA (*mcl*-PHA) composed of 6 to 14 carbon atoms. Physico-chemical properties differed between *scl*-PHA, that are rigid and brittle polymers and *mcl*-PHA that are usually more rubbery and ductile (Pérez-Rivero and Hernandez-Raquet, 2017). Poly(3-hydroxybutyrate) (PHB) is one of the most widespread and best characterized among the PHA. With high crystallinity (>50%), it is a relatively brittle and stiff polymer (Corre et al., 2012; Koller et al., 2010). Production of the copolymer with valeric acid triggers the incorporation of 3-hydroxyvalerate (HV) and results in the less stiff and brittle copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV), easier to process for commercial applications (Lemechko et al., 2019; Pachekoski et al., 2009). *Mcl*-PHA display properties that could replace elastomers. They are rubbery, soft and showed low degree of crystallinity, melting temperature and glass transition (Abe et al., 2012). Despite the recent interest of their properties for biomedical or cosmetic applications, *mcl*-PHA are not produced in industrial quantities and the relationships between their biodegradation and their physico-chemical properties has been poorly explored (Abe et al., 2012).

Biofilm growing on plastic are characterized by very diverse and niche-specific microbial communities called “plastisphere” (Zettler et al., 2013) that can play a predominant role in plastic degradation (Jacquin et al., 2019). Previous studies showed the great abilities of microbial communities to biodegrade *scl*-PHA in the marine environment (Deroiné et al., 2014; Volant et al., 2022). The microbial communities colonizing commercial PHA (PHBHV) under marine conditions have been studied during semi- and long-term colonization, but no clear

relation was made with biodegradation (Dussud et al., 2018; Odobel et al., 2021). Due to the weak commercial availabilities, no studies have ever reported microbial activity and diversity on *mcl*-PHA, thus resulting in a lack of comparison between the environmental “end of life” of plastics made of *scl*- or *mcl*-PHA families.

In this study, we describe the bacterial production of 6 different tailor-made *scl*- and *mcl*-PHA by *Halomonas sp. SF2003* (Thomas et al., 2019) and *Pseudomonas putida KT2440* (DSM 6125), respectively, together with their physico-chemical characterization. We also analysed their biodegradation by using a two steps protocol including a one-month pre-colonisation step for each PHA group (*scl*- and *mcl*-PHA) and controls (cellulose and Polyethylene, PE) to mimic the growth of marine natural biofilms and another step in minimum medium with plastics as sole carbon source to test biodegradation. We hypothesized that various PHA types and their related chemical and physical properties, as well as the associated natural biofilms are driving the biodegradation activities in seawater. We used a multidisciplinary approach to produce (bioreactor) and characterize six tailor-made PHA (gas chromatography, steric exclusion chromatography, contact angles, differential scanning calorimetry) and to evaluate the bacterial diversity (16S rDNA Illumina sequencing) associated to the biodegradation activity (oxygen consumption, heterotrophic bacterial production) of each polymer type.

## 2. Materials and methods

### 2.1. Production of *scl*- and *mcl*-PHA

Pre-culture of *Halomonas sp. SF2003* for *scl*-PHA production and *Pseudomonas putida* KT2440 for *mcl*-PHA production were both performed in 500 mL Erlenmeyer flasks at 30°C and 200 rpm, with incubations for 8h in Zobell media (Thomas et al., 2019) or for 16h in mineral medium (Maclean et al., 2008), respectively. The preculture was then transferred into a 5 L bioreactor (GPC-BIO, MINIPROLAB, France) containing a final volume of 2 L of Zobell medium or mineral medium for *scl*- and *mcl*-PHA production, respectively. Cultivation temperature was 30 °C, pH  $7 \pm 0.2$ . Agitation was at a minimum of 400 rpm to maintain a dissolved oxygen concentration above 30%, as measured with optical dissolved oxygen sensors (Hamilton company, Switzerland).

Prior to PHA accumulation from different carbon sources, *scl*-PHA fermentation started with 10 g.L<sup>-1</sup> of glucose to promote growth. After 12h, one pulse of 5 g.L<sup>-1</sup> of glucose was added every 4 hours until 24 h for the PHB accumulation. A mix of glucose and valeric acid

(50/50, % mol) was continuously dropped in the bioreactor at a rate of 4 mL.h<sup>-1</sup> for 24 h for the poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV6) fermentation. A mix of glucose and valeric acid (70/30, % mol) was continuously dropped at a rate of 6 mL.h<sup>-1</sup> for 24 h for the PHBV11 fermentation. *Mcl*-PHA fermentation also started with glucose implementation to promote high cell density before PHA accumulation, as previously described (Sun et al., 2006). Briefly, it included a first phase of 24h growth with a feeding strategy based on pulses of exponential quantity of glucose during 7h (12.5 g.L<sup>-1</sup> of glucose) followed by a linear feeding strategy from 7 to 24h by adding a pulse of 1 g of glucose additionally to the previous quantity of glucose pulsed every hour. After 24 h, 1 g of fatty acids (octanoic, heptanoic or nonanoic acid to produce a poly(3-hydroxyoctanoate) (PHO), a poly(3-hydroxynonanoate) (PHN) and another PHN called “PHNac”, respectively) was added when the dissolved oxygen concentration was above 30% (approximately every 15 minutes).

At the end of the fermentation, bacterial cells were recovered by centrifugation and PHA were separated from bacterial biomass using an incubation with an excess of solvent (50 mL of chloroform for approximately 1 g of *scl*-PHA at 60°C overnight and 40 mL of dichloromethane at room temperature overnight for approximately 1 g of *mcl*-PHA). For *scl*-PHA extraction, distilled water (V/V) was added after cooling down. The suspension was mixed and centrifuged to recover the organic layer before filtration on glass cotton and casting in a glass Petri dish. *Mcl*-PHA solution were filtered on a 1.2 µm glass microfibers paper and the concentrated *mcl*-PHA solution was precipitated in cold ethanol (10% v/v) and stored at 6 °C for two days before collection. Casting process consisted in dissolving PHA in their respective solvents, pouring PHA solutions into glass Petri dish covered with their lids that were opened briefly twice a day until constant weight to allow slow solvent evaporation. Films were stored at room temperature and in the dark.

## 2.2. Physico-chemical characterization of the six tailor-made PHA

Compositions of the produced PHA were determined by gas chromatography (GC). *Scl*-PHA and *mcl*-PHA underwent a propanolysis (Riis and Mai, 1988) and a methanolysis (Furrer et al., 2007), respectively. Briefly, 10 mg of PHA were dissolved in 1 mL of chloroform or dichloromethane and further propyl esterified (*scl*-PHA) with 1 mL of a solution of 1-propanol/37% HCl (8/2, V/V) or methyl esterified (*mcl*-PHA) with 1 mL of MeOH/BF<sub>3</sub> (10% BF<sub>3</sub>, V/V) at 80°C for 20h. After cooling down, distilled water (V/V) was added and the solutions were vortexed. The organic phase was retrieved, dried on MgSO<sub>4</sub>, filtered on glass cotton and samples were injected on a Perkin Elmer Clarus 480 gas chromatograph equipped

with a 30 m x 0.32 mm DB–5 column (HP) with splitless injector and flame ionization detector (FID). Oven temperature, ramp and nitrogen flow were measured according to Riis and Mai (1988) and Furrer et al. (2007).

Molecular weights were measured by steric exclusion chromatography (SEC) using an Agilent Technologies 1200 Infinity II containing an isocratic pump, a column oven at 35 °C and a RI detector. PHA sample separations were performed by two columns PLgel (Mixed-E, 3 µm and Mixed-D, 5 µm) from Polymer Laboratories for *scl*-PHA and by two columns from Malvern Panalytical technologies (LT4000L, 4µm and LT5000L, 10 µm) for *mcl*-PHA and a column guard. About 10 mg of PHA were first dissolved into 2 mL of chloroform (*scl*-PHA) or THF (*mcl*-PHA) then filtered with PTFE filter (0,45 µm) before a 50 µL injection.

Contact angles were measured on each PHA using a drop shape analyser from KRÜSS scientific technologies with a 2 µL droplet of water placed on the air side surface of the films that have been previously stabilized for 3 weeks at room temperature. More than 5 measurements were carried out for a single sample and the resulting values were averaged.

Thermal properties were determined by differential scanning calorimetry (DSC) using a Mettler-Toledo DSC-882 equipment. About 6 mg of PHA were taken from three weeks old casted-films of PHA. Particular attention was given so that all the PHA samples get the same thermal history in order to avoid the induction of different structures, as reported elsewhere (Crétois et al., 2016; Laycock et al., 2014; Xie et al., 2008). PHA samples were equilibrated to 25°C then heated to 190 °C for *scl*-PHA or 80°C for *mcl*-PHA at 10 °C.min<sup>-1</sup> and kept isothermal for 2 min followed by a cooling down to -40 °C for *scl*-PHA or -70°C for *mcl*-PHA at 10 °C.min<sup>-1</sup>. A second isotherm at -40°C or -70°C was kept for 2 min then the sample was heated from -40 to 190 °C or -70°C to 80°C at 10°C.min<sup>-1</sup> according to the PHA type. Melting temperatures and melting enthalpies were measured from the second heating ramp for *scl*-PHA while on the first heating ramp for *mcl*-PHA. Glass transition temperatures were measured on the second heating ramp for all PHA and values correspond to the inflection point.

### 2.3. Experimental setup of the biodegradation assay

A two phase stepwise experiment was designed in order to evaluate the biodegradability of the polymers under marine conditions, as previously described (Cheng et al., 2022). Briefly, the first step consisted of the formation of a mature biofilm on each PHA groups (PHBV colonization for the *scl*-PHA group including PHB, PHBV6 and PHBV11; PHO colonization for *mcl*-PHA including PHO, PHN and PHNac) together with a positive control (Cellulose colonization, CELLU, Whatman 42) and a negative control (Polyethylene



colonization, PE, Symphony Environmental Technology, UK). Large rectangular pieces of 13.5 cm<sup>2</sup> of each polymer type were incubated for one month (5 August to 6 September 2021) in separate 2.4L aquarium with continuous circulating seawater (flow rate ranged from 8 to 12 ml.min<sup>-1</sup>) pumped in the Banyuls bay (NW Mediterranean Sea). Throughout the experiment, seawater temperature (between 19°C and 24°C) and salinity (38.5 g.L<sup>-1</sup>) in the aquarium were similar to seawater from the Banyuls bay. Secondly, individual biofilms were detached from two pieces of each PHA or PE or Cellulose colonized films by three cycles of 1 min vortex and 3 min sonication and resuspended in a 40 mL of carbon-minimum medium called “MM” hereafter (Appendice). Cell counts was verified by flow cytometry (FACSCanto II flow cytometer, BD Bioscience, San Jose, CA) and adjusted to 10<sup>5</sup> cells.mL<sup>-1</sup>. Three discs of 6 mm<sup>2</sup> diameter each (56.5 mm<sup>2</sup> total surface) of each PHA, PE and Cellulose were placed in 12 mL Exetainer tubes (Exetainer flat bottom 12 mL, Labco, Lampeter, UK) together with 3 mL of the corresponding inoculum previously detached. The tubes were incubated in the dark at 18°C under agitation at 110 rpm (orbital agitator, Innova® S44i, Eppendorf, Germany) for a 2-month period (called “biotic conditions” hereafter). In addition, similar incubation and sampling procedure were used for abiotic controls, which consisted of triplicate vials containing 3 mL of MM with plastics of the same composition (called “abiotic condition” hereafter). A total of 887 tubes were needed to follow the different parameters detailed below, with triplicates samples taken after 0, 1, 15, 30 and 60 days of incubation.

#### 2.4. Continuous oxygen measurement

During the second step of the experiment, duplicate vials with each plastic type were equipped with an optical fiber luminescent oxygen sensor (SP-PSt5, Presens, Germany) using a small 24-channel reader (SDR SensorDish®, Presens, Germany). Oxygen sensors were placed in the liquid phase to obtain the concentration of dissolved oxygen recorded every hour during 60 days.

#### 2.5. Heterotrophic Bacterial Production

Heterotrophic Bacterial Production (BP) was measured on triplicate sample for each PHA type at 15, 30 and 60 days by <sup>3</sup>H-leucine incorporation into proteins, as previously described (Dussud et al., 2018). Briefly, a soft cell detachment pre-treatment based on three cycle of vortex and sonication was first performed. Then, <sup>3</sup>H-leucine (specific activity of 112 Ci.mmol<sup>-1</sup>) was added on PHA samples (final concentration of 1 nmol.L<sup>-1</sup> after addition of cold leucine). Radioactivity was measured using a Beckman Scintillation Counter (LS 5000CE) after

addition of TCA 50% and resuspension in a liquid scintillation cocktail (Ultima Gold). Empirical conversion factor of 1.55 ng C.pmol<sup>-1</sup> of incorporated leucine was used to calculate BP (Simon and Azam, 1989). Blanks followed the same protocol but bacterial activity was stopped by the introduction of 50% TCA prior to the addition of the radioactive mix.

## 2.6. DNA extraction and sequencing.

Plastic pieces were sampled at 15, 30 and 60 days and stored at -80°C until DNA extraction. One liter seawater was sampled from the control aquarium, successively filtered onto 3-µm and 0.2-µm pore size polycarbonate filters (PC, 47 mm diameter, Nucleopore), and filters were stored at -80°C. We also sampled the initial biofilm previously detached after the first one-month colonisation step on PHBHV, PHO, cellulose and PE films, which was stored at -80°C after filtration onto 0.2-µm pore size polycarbonate filters (PC, 47 mm diameter, Nucleopore). DNA extractions were realized on all samples using the same phenol-chloroform method, as previously described (Odobel et al., 2021). Primers used for PCR amplification of the 16S V3–V5 region were 515F-Y and 926R (Fuhrman et al., 1989), previously shown as well-suited for marine samples (Parada et al., 2016). Sequencing was performed on Illumina MiSeq by Genoscope (Evry, France), generating 3,060,721 paired sequences in the 29 samples. Raw FASTA files were deposited at EBI under the accession number ERP148254. Sequence analysis was processed using the package DADA2 (Version 1.24.0) into R studio software (R Core Team, 2022, version 4.2.2). Standard pipeline was applied with the following parameters: trimLeft= c(19,20), truncLen= c(240,240), maxN=0, maxEE=c(2,2), truncQ=2. The sequences were therefore filtered, dereplicated, denoised by removing sample interference and chimeras before merging. Clusters were assigned with the Silva 128 16S rRNA database (Quast et al., 2013) and clusters that did not belong to Bacteria kingdom were removed as well as chloroplast and mitochondrial sequences. The number of sequences per sample was normalized by rarefaction (n= 21,324) and a table with 29 samples and 5,053 amplicon sequence variants (ASV) was obtained.

## 2.7. Statistical analysis

All graphical representations and statistical analysis were performed on R studio software (R Core Team, 2022, version 4.2.2) using the packages ggplot2 (Wickham, 2016), vegan (Oksanen et al., 2007) and phyloseq (McMurdie and Holmes, 2012) and PRIMER6 (Clarke and Gorley, 2006). Data were compared with Kruskal-Wallis tests followed by post hoc tests at D60 for oxygen consumption and heterotrophic bacterial production. Sequences

were analyzed with the phyloseq package. The alpha diversity indexes were calculated and compared with Wilcoxon tests. Differences in microbial community structure among samples were tested by ANOSIM based on Bray-Curtis distances (PRIMER6 software). The ASVs that contributed most to differentiate microbial community structures between *scl*-PHA vs. PE, *scl*-PHA vs. *mcl*-PHA and *mcl*-PHA vs. PE were tested with a similarity percentage analysis (SIMPER, PRIMER6) (Clarke, 1993).

### 3. Results

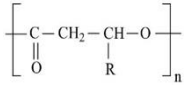
#### 3.1. Physico-chemical characterization of the six tailor-made PHA

Three fermentation processes in bioreactor with *Halomonas sp. SF2003* growing in different substrates resulted in the production of three *scl*-PHA: PHB, PHBHV6 and PHBHV11 (Table 1). *Halomonas sp. SF2003* growing on glucose accumulated a homopolymer of PHB composed at 100% of 3-hydroxybutyrate units (HB) (Table 1) with 4 carbons per monomer. A mix of glucose and valeric acid (70/30, % mol) resulted in PHBHV11 production composed of 89% of HB and 11% of HV, leading to an average number of 4.11 carbons per monomer. PHBHV6 was produced using another mix of glucose and valeric acid (50/50, % mol) resulting in a copolymer composed of 94% of HB and 6% of HV with an average number of 4.06 carbons per monomer. Fermentations from *Pseudomonas putida KT2440* from two different fatty acids (octanoic and nonanoic acid) and a beta-oxidation pathway inhibitor (acrylic acid) (Jiang et al., 2013) led to three *mcl*-PHA: PHO mainly composed of 3-hydroxyoctanoate (HO) monomer (89%) then 3-hydroxyhexanoate (HHx) and 3-hydroxydecanoate (HD) (5.5% each) for an average number of 7.78 carbons per monomer, PHN composed of 3-hydroxynonanoate (HN) monomer units (58%) then composed of HD (24%), 3-hydroxyheptanoate (HHp) (14%) and HO (4%) units with an average number of 8.92 carbons per monomer and PHNac composed of HN (73%), HHp (23%) and HD (2%) with an average number of 8.47 carbons per monomer.

Thermal properties of all PHA were characterized by DSC (Table 1). *Scl*-PHA displayed glass transition temperatures between -7°C and 9°C. *Scl*-PHA with HV units (PHBHV6 and PHBHV11) showed several lower peaks of melting temperatures (129 and 154°C for PHBHV6 and 122, 129 and 147°C for PHBHV11) compared to PHB (161°C). Melting enthalpies also differ according to the chemical composition. Higher melting enthalpy is observed for PHB ( $\Delta H_m = 87 \text{ J.g}^{-1}$ ) then followed by PHBHV6 ( $\Delta H_m = 77 \text{ J.g}^{-1}$ ) and finally by PHBHV11 ( $\Delta H_m = 40 \text{ J.g}^{-1}$ ) due to the presence of HV units which partially limit the chain crystallisation. Number average molecular mass could not be measured for PHB due to

solubility difficulties. PHBHV6 and PHBHV11 displayed  $\overline{M}_n$  of 340 000 and 325 000 g.mol<sup>-1</sup> with a polydispersity index (PDI) of 2.8 and 2.9, respectively. Among *scl*-PHA, contact angles with distilled water revealed that PHB is a less hydrophobic polymer with a contact angle of 64 ± 1.6°, followed by PHBHV11 (74 ± 0.3°) and PHBHV6 (76 ± 0.3°).

**Table 1:** Composition of the 6 tailor-made PHA and their associated thermal characteristics, average molecular mass and contact angle.

	Chemical composition	Average number of carbon per monomer	$\overline{M}_n$ (g.mol <sup>-1</sup> )	PDI	T <sub>g</sub> (°C)	T <sub>m</sub> (°C)	ΔH <sub>m</sub> (J.g <sup>-1</sup> )	Contact angle (°)
<b>PHB</b>	100% HB	C <sub>4.00</sub>	-	-	9	161	87	64 ± 1.6
<b>PHBHV6</b>	94% HB 6% HV	C <sub>4.06</sub>	340 000	2.8	-7	129 154	77	76 ± 2.4
<b>PHBHV11</b>	89% HB 11% HV	C <sub>4.11</sub>	325 000	2.9	-7	122 129 147	40	74 ± 0.3
<b>PHO</b>	5.5% HX 89% HO 5.5% HD	C <sub>7.78</sub>	84 000	2.2	-36	57	20	82 ± 1.8
<b>PHN</b>	14% HHp 4% HO 58.1% HN 24% HD	C <sub>8.92</sub>	60 000	2.7	-40	49	20	90 ± 2.4
<b>PHNac</b>	23% HHp 74% HN 2% HD	C <sub>8.47</sub>	70 000	2.2	-39	52	13	80 ± 0.6

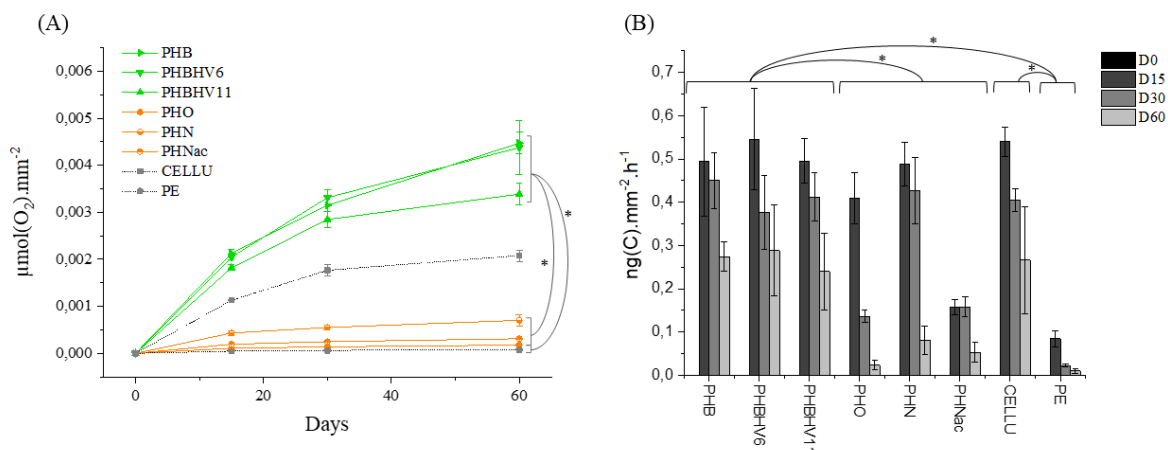
On the other hand, *mcl*-PHA displayed lower glass transition temperatures (PHO: -36°C, PHN: -40°C and PHNac: -39°C) and lower melting temperatures (PHO: 57°C, PHN: 49°C and PHNac: 52°C) than all *scl*-PHA. Melting enthalpies is also far lower than *scl*-PHA with a melting enthalpy of 20 J.g<sup>-1</sup> for PHO and PHN and a lower one of 13 J.g<sup>-1</sup> for PHNac.  $\overline{M}_n$  of *mcl*-PHA ranged from 60 000 to 84 000 g.mol<sup>-1</sup> with PDI ranging from 2.2 to 2.7. High

hydrophobicity is displayed by *mcl*-PHA, especially for PHN ( $90 \pm 2.4^\circ$ ) then for PHO and PHNac ( $82 \pm 1.8^\circ$  and  $80 \pm 0.6^\circ$ , respectively). Overall, physico-chemical properties of the PHA produced differed greatly according to the type of PHA (*scl*- or *mcl*-) while slight but noticeable differences were found within both PHA type.

### 3.2. Biodegradation activities

Several parameters were used to evaluate the biodegradability of the various PHA. First, continuous oxygen consumption (Presens sensors) by microorganisms with PHA as sole carbon and energy source showed clear distinction between *scl*- and *mcl*-PHA. During the first 15 days, oxygen consumption rapidly increased and tended to reach a plateau from 15 to 60 days for the *mcl*-PHA while it kept increasing to a greater extent for *scl*-PHA (Fig. 1A). At day 60, PHB (mean =  $4.5 \pm 0.23 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$ ), PHBHV6 (mean =  $4.4 \pm 0.57 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$ ), and PHBHV11 ( $3.4 \pm 0.2 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$ ) presented a much higher oxygen consumption than PHO ( $0.16 \pm 0.018 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$ ), PHN ( $0.70 \pm 0.11 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$ ), and PHNac ( $0.31 \pm 0.019 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$ ). Significant difference was found between *scl*- and *mcl*-PHA biodegradation ( $p < 0.05$ ), as well as with between *scl*- and the PE control ( $0.072 \pm 0.0092 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$ ,  $p < 0.05$ ). No significant difference was found between the oxygen consumption on *scl*-PHA compared to Cellulose ( $2.1 \pm 0.12 \times 10^{-3} \mu\text{mol}(\text{O}_2).\text{mm}^{-2}$ ,  $p > 0.05$ ). Likewise, kinetics comparison intra *scl*-PHA or intra *mcl*-PHA did not show any statistical differences. Among *mcl*-PHA, small but noticeable oxygen consumption was observed for PHN, while PHO and PHNac had a similar trend to the PE negative control.

Second, cell incorporation of  $^3\text{H}$ -leucine into proteins showed similar trends, with significantly higher heterotrophic activities for *scl*-PHA as compared to *mcl*-PHA. Within the first 15 days of incubation in minimum medium, the activities of the biofilms were high and then decreased until day 60 for all the PHA (Fig. 1B). At day 60, maximum activities were found for the positive control cellulose ( $2.7 \pm 1.2 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$ ) and *scl*-PHA, including PHBHV6 ( $2.9 \pm 1.0 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$ ), PHB ( $2.8 \pm 0.34 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$ ) and PHBHV11 ( $2.4 \pm 0.89 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$ ). Much lower bacterial activities were observed for the negative control PE ( $0.099 \pm 0.060 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$ ) and *mcl*-PHA, including PHN ( $0.81 \pm 0.32 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$ ), PHNac ( $0.53 \pm 0.022 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$ ), PHO ( $0.23 \pm 0.11 \times 10^{-1} \text{ng}(\text{C}).\text{mm}^{-2}.\text{h}^{-1}$ ), that significantly differed from cellulose ( $p < 0.05$ ).



2

3 **Figure 1:** Oxygen consumption (A) and bacterial heterotrophic production (B) on the different polymers  
 4 (PHB, PHBV6, PHBV11, PHO, PHN and PHNac) in minimum media for 15, 30, 60 and 90 days.  
 5 Errors bars indicate standard deviation. \* indicates significant difference by Kruskal-Wallis test on day  
 6 60.

### 7 3.3. Bacterial diversity

8 During the 60-days of experiment, no significant change in alpha-diversity were  
 9 observed in all polymer type over time, including all the measured diversity indexes (Chao1  
 10 richness, Pielou evenness, Shannon and Simpson diversity) ( $p > 0.05$ ) (Table 2). However,  
 11 significant differences were found between the polymer groups, including cellulose and *scl*-  
 12 PHA (PHB, PHBV6 and PHBV11), as compared to another group including PE and *mcl*-  
 13 PHA (PHO, PHN and PHNac) ( $p < 0.05$ ). Lower Chao1 richness and Shannon diversity were  
 14 found for *scl*-PHA ( $244.4 \pm 8.8$  and  $3.3 \pm 0.3$ ;  $n = 9$ , respectively) as compared to *mcl*-PHA  
 15 ( $606.8 \pm 19.4$  and  $4.4 \pm 0.4$ ;  $n = 9$ , respectively) ( $p = 1.6 \times 10^{-4}$ ). Higher diversity on the free-  
 16 living bacteria and on the initial inocula for each polymer type was also observed (Table 2).

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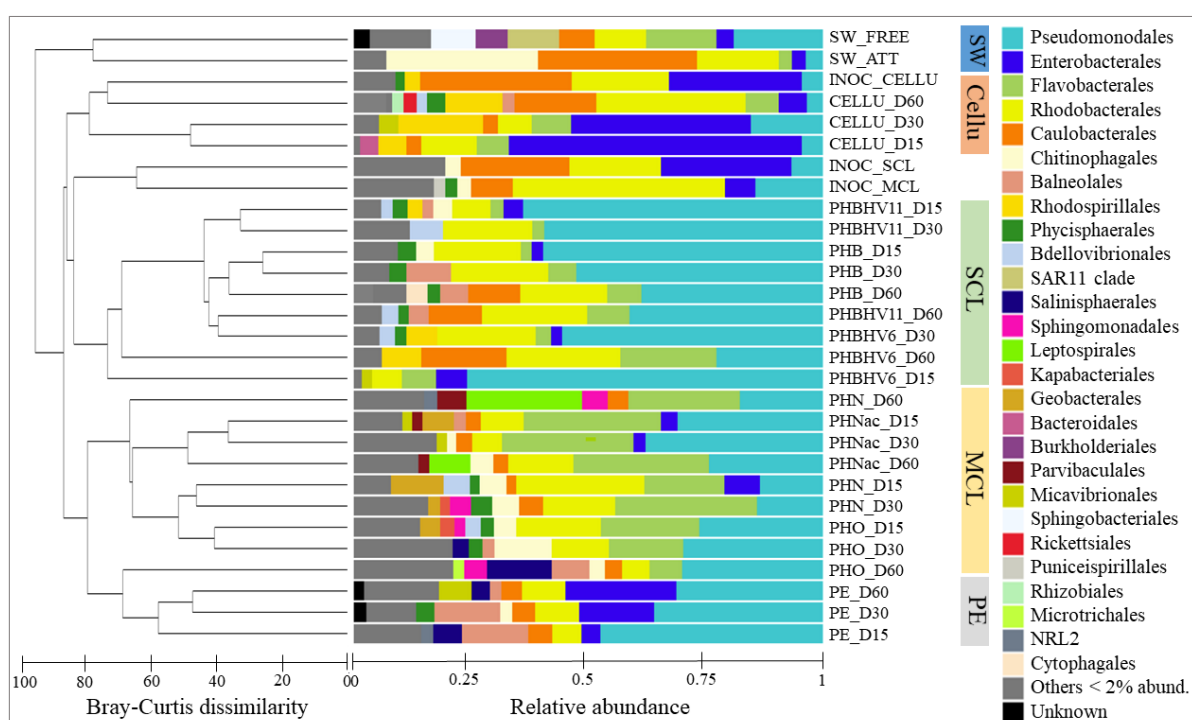
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25 **Table 2:** Total number of ASV per sample together with *Chao1* richness, Pielou evenness and Shannon,  
 26 and Simpson diversity indexes. SW: seawater, INOC: microbial inoculum from each polymer types after  
 27 one month of colonization, D: incubation time in days.

Sample	Total ASV	<i>Chao1</i>	Pielou	Shannon	Simpson
SW-FREE	939	1313	0.716	4.9	57.8
SW-ATT	497	541	0.644	4	18.3
INOC_CELLU	707	889	0.671	4.4	25.5
CELLU_D60	207	226	0.656	3.5	15.5
CELLU_D30	288	322	0.636	3.6	12.7
CELLU_D15	128	129.	0.556	2.7	5.8
INOC_SCL	651	656.	0.818	5.3	75.4
INOC_MCL	1276	1425	0.755	5.4	42.9
PHBHV11_D15	307	331	0.559	3.2	8.2
PHBHV11_D30	166	171	0.548	2.8	6.3
PHB_D15	313	343	0.574	3.3	11.4
PHB_D30	243	264	0.564	3.1	11.4
PHB_D60	213	246	0.653	3.5	18
PHBHV11_D60	266	294	0.591	3.3	10.9
PHBHV6_D30	212	237	0.672	3.6	14.8
PHBHV6_D60	156	163	0.733	3.7	27
PHBHV6_D15	147	148	0.561	2.8	8
PHN_D60	423	528	0.678	4.1	23.5
PHNac_D15	435	470	0.691	4.2	23.8
PHNac_D30	517	560	0.640	4	14.5
PHNac_D60	403	472	0.650	3.9	20.7
PHN_D15	434	498	0.692	4.2	25.4
PHN_D30	290	302	0.776	4.4	45.3
PHO_D15	689	767	0.719	4.7	48.7
PHO_D30	753	879.	0.740	4.9	58.4
PHO_D60	822	983	0.775	5.2	74.7
PE_D60	711	828	0.701	4.6	31.3
PE_D30	616	676	0.747	4.8	65.5
PE_D15	547	614	0.793	5	82.7

28

29 Beta-diversity analysis showed four distinct groups between bacterial communities  
 30 living on cellulose, *scl*-PHA (PHB, PHBHV6 and PHBHV11), *mcl*-PHA (PHO, PHN and  
 31 PHNac), PE and in seawater (Fig. 2). Interestingly, inoculum grown on cellulose before the  
 32 experiment grouped with the bacterial communities living on cellulose as sole carbon source.  
 33 It was not the case for *scl*-PHA and *mcl*-PHA inocula, that changed when incubated with the  
 34 different polymer types. ANOSIM analysis showed significant differences between *scl*-PHA  
 35 and *mcl*-PHA samples ( $R = 0.964$ ,  $p < 0.05$ ). Within these groups, no clear distinction could be  
 36 made between samples, except for PHBHV6 (day 15 and 60) for *scl*-PHA and PHO-D60 for  
 37 *mcl*-PHA.



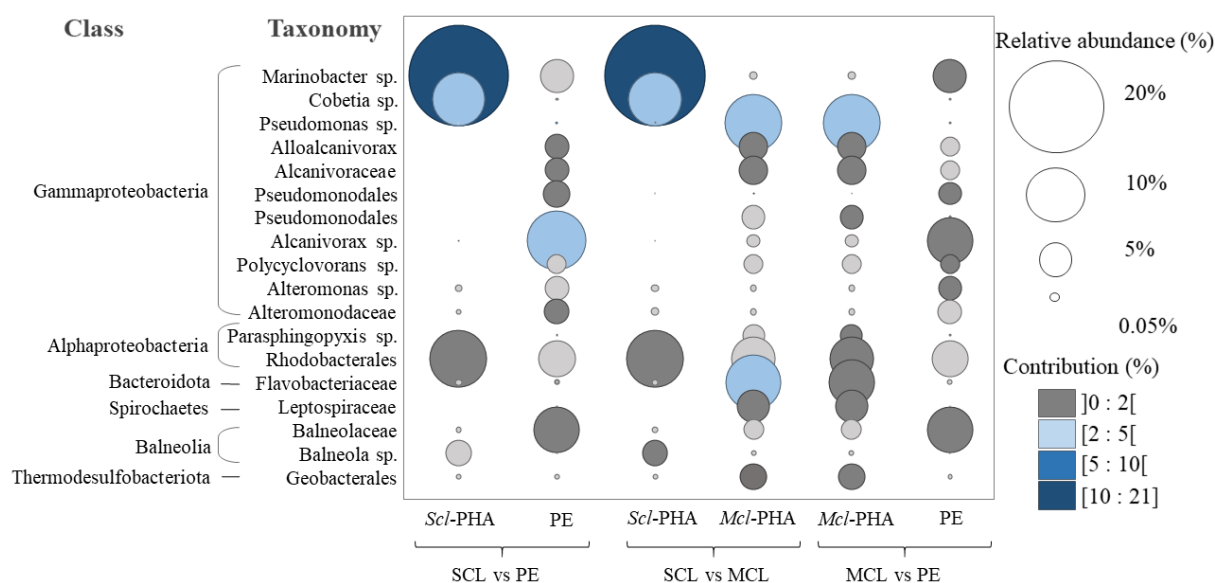
38  
 39 **Figure 2:** Comparison of community structures and taxonomic abundances of bacteria in seawater  
 40 (SW), on the microbial inoculum from each polymer types after one month of colonization (INOC) and  
 41 bacteria on polymers (PHB, PHBHV6, PHBHV11, PHO, PHN and PHNac) according to time (in days,  
 42 D), by UPGMA dendrogram based on Bray-Curtis dissimilarities between sequencing profiles (left) and  
 43 by cumulative bar charts comparing relative order abundances (right).



Taxonomic composition confirmed the niche-partitioning between the bacterial communities living in seawater compared to the plastisphere of the different polymer types. Free-living bacteria were composed of Pseudomonadales (19%), Flavobacteriales (15%), Rhodobacterales (11%), SAR11 clade (11%), Sphingobacteriales (9%) and Caulobacterales (8%), while organic particle-attached bacteria were dominated by Caulobacterales (34%), Chitinophagales (32%) and Rhodobacterales (17%). The inoculum pre-grown on cellulose in seawater was mainly composed of Caulobacterales (32% for INOC\_CELLU), Rhodobacterales (21% for INOC\_CELLU), Enterobacterales (28% for INOC\_CELLU) and to a lesser extent of Pseudomonadales (5% for INOC\_CELLU). The same groups were also found for the inocula pre-grown on *scl*-PHA and *mcl*-PHA but with different proportions, including Caulobacterales (23% for INOC\_SCL and 9% for INOC\_MCL), Rhodobacterales (20% for INOC\_SCL and 45% for INOC\_MCL), Enterobacterales (28% for INOC\_SCL and 7% for INOC\_MCL) and Pseudomonadales (5% for INOC\_CELLU, 7% for INOC\_SCL and 14% for INOC\_MCL). After inoculation with the different polymer type as sole carbon source, the proportion of different taxa was different between the different polymer types. Pseudomonadales were high on *scl*-PHA ( $52 \pm 15\%$ , n=9), *mcl*-PHA ( $25 \pm 2\%$ , n=9), PE samples ( $28 \pm 7\%$ , n=3) and cellulose ( $8 \pm 5\%$ , n=3) together with Rhodobacterales (mean =  $18 \pm 6\%$ ,  $13 \pm 7\%$ ,  $8 \pm 1\%$ ,  $17 \pm 10\%$ , n=9 for *scl*-PHA, *mcl*-PHA, PE and cellulose, respectively). *Mcl*-PHA exhibited high proportions of Flavobacteriales (mean =  $22 \pm 7\%$ , n = 9) compared to other polymers (between 0% on PE and 10% on others polymer). Main taxa found on cellulose was Enterobacterales (mean =  $35 \pm 23\%$ , n = 3), especially on CELLU\_J15 and CELLU\_J30 (Fig. 2).

SIMPER analysis on the Bray Curtis dissimilarity highlighted two species contributing to the differences between *scl*-PHA and PE. First, ASV affiliated to *Marinobacter sp.* showed a high relative abundance in *scl*-PHA (more than 37%) and a high contribution (20%) (Fig. 3) compared to PE. *Cobetia sp.* is the second most specific species found on *scl*-PHA in a lesser abundance (10%) and contributes to 6% on *scl*-PHA. Those two species that display a clear distinction between *scl*-PHA and PE microbial communities also contributed to the differences found between *scl*-PHA and *mcl*-PHA. Indeed, *Marinobacter sp.* and *Cobetia sp.* were poorly represented on *mcl*-PHA, while major relative abundance and contribution were found for *Pseudomonas sp.* (12% and 7% for relative abundance and contribution, respectively) and Flavobacteriaceae (11% and 6% for relative abundance and contribution, respectively) for this polymer type. Additionally, *Pseudomonas sp.* and Flavobacteriaceae were poorly represented on *scl*-PHA, as well as on PE. Bacterial communities observed on *mcl*-PHA and PE seemed richer and more scattered. With the exception of Rhodobacterales that were abundant on all polymer types, taxa belonging to *Alcanivorax sp.* (13% and 8% of relative abundance and contribution, respectively), Balneolaceae, Pseudomonadales,

Alloalcanivorax, Alcanivoraceae and Alteromonadaceae found on PE were poorly abundant on *scl*-PHA. Taxa belonging to Alloalcanivorax and Alcanivoraceae were also present on *mcl*-PHA but presented low contribution (2%) when compared to PE.



**Figure 3:** Bubble plot showing the relative abundance and the taxonomy of the majority ASV contributing to 50% of the dissimilarity between *scl*-PHA vs. *mcl*-PHA, *scl*-PHA vs. PE and *mcl*-PHA vs. PE. Points are sized according to the relative abundance and colored by their contribution to dissimilarity.

## 4. Discussion

### 4.1. Different physico-chemical characteristics of the six tailor-made PHA

The first step of this study was to produce three *scl*-PHA and three *mcl*-PHA with various intrinsic properties in order to estimate the influence of the physico-chemical characteristics in the PHA biodegradation in the marine environment. The bacterial strain *Halomonas sp.* SF2003 has been used for the production of *scl*-PHA using glucose and/or valeric acid to generate PHB, PHBV6 and PHBV11. Surprisingly, an increase of valeric acid in the substrate feeding led to a lower HV content in PHBV6. This conducted to the permanent presence of low valeric acid concentration in the bioreactor but also glucose concentration. *Halomonas sp.* SF2003 cells seem, in this particular conditions, metabolize glucose for HB accumulation at the expense of valeric acid and therefore HV incorporation in the polymer. The use of different substrates induced different chemical compositions that affect thermal properties. Glass transition temperatures changed from  $-7^{\circ}\text{C}$  to  $9^{\circ}\text{C}$  with only 6% of HV incorporation. Melting temperatures dropped from 161 to  $154^{\circ}\text{C}$  and  $147^{\circ}\text{C}$  with 6% and 11% of HV incorporation, respectively. Overall, DSC analysis showed common features found in other *scl*-PHA produced and characterized in the literature (Koller et al., 2010; Lemechko et al., 2019;

Możejko-Ciesielska and Kiewisz, 2016). The melting enthalpy, attributed to the PHA crystallinity, was a parameter highly influenced by the HV incorporation due to a higher steric hindrance generated by the HV units compared to that of HB units. It tended to decrease as much as the HV content increased to reach  $40 \text{ J.g}^{-1}$  with 11% of HV unit content. The modulation of HV unit proportion, even at low incorporation, is a promising way to modify the PHA properties, for instance to bring softness and elasticity to the homopolymer PHB, which can show some difficult features to process due to its high crystallinity and a melting temperature close to its degradation temperature (Pachekoski et al., 2013). Regarding *mcl*-PHA, different chain lengths and/or monomer proportions have been achieved, thus impacting to a certain extent the intrinsic properties of each of these *mcl*-PHA. PHO was mainly composed of HO (89%), PHN was mainly composed of HN (58%) and the addition of acrylic acid improved the HN content in PHNac (74%). Improving the HN content in PHNac seemed to mainly modify the crystallinity, since the melting enthalpy drops from 20 to  $13 \text{ J.g}^{-1}$  in this latter polymer. Generally, thermal properties between *mcl*-PHA slightly differ and are in accordance with PHA composed of these monomers (Abe et al., 2012; Możejko-Ciesielska and Kiewisz, 2016). Tailor-made production with different strains and carbon sources allowed to produce PHA displaying clear and significant distinctions between *scl*- and *mcl*-PHA, including thermal properties, molecular weights or hydrophobicity. Differences within PHA types were slighter but still noticeable, especially for the crystallinity difference observed within the *scl*-PHA types.

#### 4.2. Various biodegradation activities on the different polymer types

The main originality of our study is to present pioneer results of microbial biodegradation activities of the various tailor-made PHA under natural marine conditions. Oxygen consumption measurements and heterotrophic bacterial production are important signals of the biodegradation potential of a polymer (Jacquin et al., 2019). Most of the proofs of PHA biodegradability in marine environment focused previously on weight loss (Deroiné et al., 2015, 2014; López-Ibáñez and Beiras, 2022; Volova et al., 2011). Weight loss provides a proof of the plastic disintegration only, which may or may not be associated to the complete mineralization by bacteria (Haider et al., 2019). It has been previously shown that PHA biodegradation undergoes an erosion process and that the shape and the size of the samples greatly impact the process (Cheng et al., 2021; Volova et al., 2010). Multidisciplinary approaches based on physical, chemical and biological parameters offer a better understanding of the biodegradation processes (Paul-Pont et al., 2023). Moreover, particular attention has been made here to produce PHA samples through the same process and of the same shape and size.

PHA being the sole carbon source in our biodegradation experiment, trend of microbial activities on *scl*-PHA clearly demonstrated their biodegradation abilities in seawater, thus confirming previous observations by using other techniques (Deroiné et al., 2015, 2014; Volant et al., 2022). Under laboratory conditions and by using pure bacterial culture, PHA biodegradation processes were depicted as the result of specialized extracellular enzymes called PHA depolymerases (Leathers et al., 2000; Mukai et al., 1993). The enzymes are capable of hydrolysing PHA chains into smaller water-soluble compounds (< 600 Da) that can cross the membranes for further bacterial degradation and assimilation (Azam and Malfatti, 2007). In our study, respiration associated to the *scl*-PHA assimilation by bacteria resulted in a regular increase in oxygen consumption during the 60 days of biodegradation tests. The respiration rates were higher than with the cellulose positive control in the same experimental conditions, whereas it was almost undetectable on PE negative control. Bacterial heterotrophic activity ( $^3\text{H}$ -Leucine incorporation) on *scl*-PHA as sole carbon source showed the same trend, with significantly higher activity on *scl*-PHA than *mcl*-PHA and PE. Both oxygen consumption and bacterial heterotrophic activities were high during the first 15 days of tests (even for PE films to a lesser extent), certainly due to the organic matter that can be detached together with the pre-colonized biofilm or due to mortality, thus rendering this period of the biodegradation tests less adequate for biodegradation measurement in our conditions. Similar difference in bacterial heterotrophic production between PHBHV and PE films was previously found during long-term colonization and biodegradation (Dussud et al., 2018; Odobel et al., 2021). Within the *scl*-PHA group, we observed slight but significantly higher oxygen consumption on PHB and PHBHV6 compared to PHBHV11 after 60 days. Such difference was not found for bacterial heterotrophic activities, rendering the difference in biodegradability within the *scl*-PHA less robust. Contrasting results found in the literature confirmed the possible but not consistent difference in biodegradation rates for these two polymers. Hypothesis of better biodegradation abilities of the PHBHV copolymer was linked to an increase of amorphous regions more susceptible to enzymatic attack compared to the homopolymer PHB (Meereboer et al., 2020; Numata et al., 2008). Other studies of *in vitro* enzymatic degradation showed the opposite, with better degradation capacities on PHB compared to PHBHV (Mukai et al., 1993). Slight differences in terms of biodegradation between *scl*-PHA are therefore difficult to explain since biodegradation is a combination of physical, chemical and biological factors (Dilkes-Hoffman et al., 2019). As a consequence, we conclude that the intrinsic differences within the *scl*-PHA properties (hydrophobicity, crystallinity, molecular weight) were not sufficient to induce a difference in biodegradation activities in our marine experimental conditions.

In the opposite, signs of biodegradation were very low or almost undetectable for the tested *mcl*-PHA types. To the best of our knowledge, this is the first study testing the biodegradability of

*mcl*-PHA, probably because no *mcl*-PHA are commercially available. The tailor-made *mcl*-PHA produced in this study showed clear distinct chemical differences between PHO, PHN and PHNac. Although PHN showed a slightly higher oxygen consumption and bacterial heterotrophic activities after 60 days than PHO and PHNac, no statistical difference was shown. It is to be noted that oxygen consumption and bacterial heterotrophic activities of the *mcl*-PHA group were similar to the negative control PE, which was a sign of very low or no biodegradability in our marine experimental conditions. We are aware that the 2-month timing of tests was probably not sufficient and we propose to perform further studies with a longer test period before giving a firm conclusion of the absence of biodegradability (in a reasonable period of time) for the *mcl*-PHA.

Interestingly, our study offers a large set of physico-chemical characteristics of various PHA that would play a key role in their biodegradability. Here, our conclusions were not in line with the classical view of the driving forces of polymer biodegradation. Indeed, physico-chemical properties such as low molecular weight, low crystallinity and low hydrophobicity were expected to facilitate polymer biodegradation (Kumar et al., 2020). We found that *scl*-PHA present higher biodegradation with higher molecular weight, crystallinity and hydrophobicity compared to *mcl*-PHA. Such discrepancy may be explained by the fact that previous conclusions were obtained from experiments under laboratory conditions with pure bacterial culture or enzyme degradation through *in vitro* experiments (Brandi et al., 1995; Numata et al., 2008; Schirmer et al., 1993; Schirmer and Jendrossek, 1994), which is very different from the environmental conditions tested in our study. Our results suggest that the chemical composition of the PHA, and more particularly the side chain structure of the polymer (*short vs. medium-chain length*) is playing a key role in the biodegradation processes in natural environment, rather than other physical parameters. Such impact of the lateral chain structure on biodegradation might result from the specificity of the extracellular PHA depolymerase to the *scl*- or to the *mcl*-PHA. Indeed, it has been previously shown that the catalytic domain activity differed between *scl*- and *mcl*-PHA depolymerase, rendering the *mcl*-PHA depolymerase ineffective on *scl*-PHA, and inversely (Kim et al., 2000). Additionally, the interesting case of the well-studied PHB-depolymerase from *Ralstonia pickettii* T1 showed a catalytic domain that would start to cleave single crystals, explaining the high biodegradability of *scl*-PHA despite their higher crystallinities (Numata et al., 2009). In the other hand, the carbon chain length of *mcl*-PHA (which present a higher molecular mobility compared to those of *scl*-PHA) could inhibit enzymatic degradation by impeding the catalytic domain with longer radical chain length and steric hindrance interferences (Numata et al., 2009).

### 4.3. Dissimilar microbial community associated to the various polymer types

Biodegradation process is a complex process involving intrinsic (relative to the polymer) but also extrinsic factors (relative to the environment). In this study, we decided to keep the same temperature, agitation, light and nutrients to focus on the impact of bacterial diversity as a key factor in plastic biodegradation. First, we put a specific attention to perform the biodegradation tests with realistic biofilm living in the natural marine environment. While there is no consensus today for the preparation of the microbial inoculum in the ISO or ASTM standards for polymer biodegradability tests, convergent views indicated that complex natural marine inoculum made of biofilm growing on the corresponding plastics under natural conditions are recommended (Cheng et al., 2022). A colonisation phase for a minimum of one month in natural seawater has been shown to be a prerequisite to mimic a mature biofilm in seawater (Jacquin et al., 2019; Odobel et al., 2021), as it has been done in this study. Bray-Curtis similarity showed that the biofilms growing during one month in natural seawater were similar in *scl*-PHA and *mcl*-PHA, but different from cellulose or PE films. As previously described in other studies, free-living and particle-attached bacteria living in the seawater presented very different communities compared to the plastisphere of the mature biofilms (Dussud et al., 2018; Wright et al., 2020), thus rendering the use of seawater as inoculum for biodegradation tests irrelevant. The transfer of pre-formed biofilm from natural seawater to minimum medium resulted in bacterial community changes for *scl*-PHA and *mcl*-PHA, but not for cellulose that remained stable during the entire 60-day incubation. Following the evolution of the bacterial community changes during the biodegradation tests has been recommended by previous studies (Jacquin et al., 2019; Kowalczyk et al., 2015), but this recommendation has been poorly followed thereafter. Changes in bacterial diversity may be used as a signal of the lack of representability of the biodegradation tests to mimic the natural environment. By following the bacterial diversity for all the tested plastics, we showed that the alpha-diversity remained stable during the course of the second step of the experiment for all plastic types, which is a prerequisite for the biodegradability tests in natural conditions (Jacquin et al., 2019).

The study of the bacterial communities also permitted to describe the potential of some ASVs to be involved in the biodegradation of the *scl*-PHA. SIMPER analysis on Bray-Curtis 16S rRNA dissimilarities showed the importance of *Marinobacter* sp. and *Cobetia* sp. in explaining the difference between the *scl*-PHA and the negative control PE. *Marinobacter* sp. has been previously shown to present abilities to degrade PHB and PHBHV (Kasuya et al., 2000; Martínez-Tobón et al., 2018). These authors demonstrated PHB and PHBHV depolymerase activities of *Marinobacter* isolated strains, and identified the *scl*-PHA depolymerase PhaZ gene. *Cobetia* sp. has never been observed as PHA-degraders, but it is a well-known producer of PHA (Christensen et al., 2021; Moriya

et al., 2020). Further studies are needed to evaluate its potential to perform both the production and the degradation of *scl*-PHA, as it has been shown for other species (Martínez-Tobón et al., 2018; Nygaard et al., 2021). These two strains were much less abundant in PE but also in *mcl*-PHA, thus suggesting a selection in *scl*-PHA associated to its biodegradation under marine conditions. Some specific species were detected in *mcl*-PHA that presented low abundance in PE. This is particularly the case for *Pseudomonas* sp., which were previously shown as very effective producers of *mcl*-PHA (Prieto et al., 2016), with the ability to also produce extracellular *mcl*-PHA depolymerase (Schirmer et al., 1993; Schirmer and Jendrossek, 1994; Young et al., 2005). *Pseudomonas* sp. was also very low in abundance in *scl*-PHA, confirming the selection of different species depending on the *scl*-PHA vs. *mcl*-PHA groups. Further long-term studies will be needed to evaluate if the *Pseudomonas* sp. selected on *mcl*-PHA may be involved in their biodegradation. This first study on *mcl*-PHA opens new routes for further studies to better understand the bacterial diversity involved in their biodegradability in the marine environment.

## 5. Conclusion

The PHA are generally cited as one solution among others to replace conventional plastics, that would be both bio-sourced and biodegradable. Most of the studies so far have proven the rapid biodegradability of *scl*-PHA that are already commercially available, but very few of them investigated the fate of *mcl*-PHA in the environment. To our knowledge, this is the first study comparing the biodegradability of *scl*- and *mcl*-PHA in the marine environment. One strength of our work was to produce six tailor-made PHA with different physico-chemical characteristics, in order to estimate the main drivers of their biodegradability in the marine environment. Overall, this study showed that the chemical nature of the polymer (*short*- vs. *medium-chain* length PHA) together with the diversity of microorganisms living on the plastic films (and probably the associated enzymes, i.e. PHA depolymerase) were the main drivers of the PHA biodegradability in the marine environment. Interestingly, we also found that the physical characteristics of the PHA (crystallinity, hydrophobicity, molecular weight) do not seem to play a limiting role in the biodegradability process within the *scl*- and *mcl*-PHA types. These results are of importance for further application of PHA with different rates of biodegradability for commercial purpose, such as the production of fishing nets, buoys or cosmetic products that end their life in the marine environment (Paul-Pont et al., 2023). This study also showed that *mcl*-PHA biodegradation takes longer time than *scl*-PHA, which could orientate the use of this PHA group for longer-lifetime products. Further biodegradation tests with longer period of time (more than 2 months) are needed to better explore the biodegradation of the

more recalcitrant *mcl*-PHA, and we believe that this study open new routes for a better understanding of *scl*-PHA and *mcl*-PHA biodegradation in the marine environment.

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## Conflict of interest:

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

## Author Contributions (CRediT taxonomy)

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## References

- Abe, H., Ishii, N., Sato, S., Tsuge, T., 2012. Thermal properties and crystallization behaviors of medium-chain-length poly(3-hydroxyalkanoate)s. *Polymer* 53, 3026–3034. <https://doi.org/10.1016/j.polymer.2012.04.043>
- Azam, F., Malfatti, F., 2007. Microbial structuring of marine ecosystems. *Nat Rev Microbiol* 5, 782–791. <https://doi.org/10.1038/nrmicro1747>
- Brandi, H., Bachofen, R., Mayer, J., Wintermantel, E., 1995. Degradation and applications of polyhydroxyalkanoates. *Can. J. Microbiol.* 41, 143–153. <https://doi.org/10.1139/m95-181>
- Cheng, J., Eyheraguibel, B., Jacquin, J., Pujo-Pay, M., Conan, P., Barbe, V., Hoypierres, J., Deligey, G., Halle, A.T., Bruzaud, S., Ghiglione, J.-F., Meistertzheim, A.-L., 2022. Biodegradability under marine conditions of bio-based and petroleum-based polymers as substitutes of conventional microparticles. *Polymer Degradation and Stability* 206, 110159. <https://doi.org/10.1016/j.polymdegradstab.2022.110159>
- Cheng, J., Jacquin, J., Conan, P., Pujo-Pay, M., Barbe, V., George, M., Fabre, P., Bruzaud, S., Ter Halle, A., Meistertzheim, A.-L., Ghiglione, J.-F., 2021. Relative Influence of Plastic Debris Size and Shape, Chemical Composition and Phytoplankton-Bacteria Interactions in Driving Seawater Plasticsphere Abundance, Diversity and Activity. *Frontiers in Microbiology* 11. <https://doi.org/10.3389/fmicb.2020.610231>
- Christensen, M., Jablonski, P., Altermark, B., Irgum, K., Hansen, H., 2021. High natural PHA production from acetate in *Cobetia* sp. MC34 and *Cobetia marina* DSM 4741T and in silico analyses of the genus specific PhaC2 polymerase variant. *Microbial Cell Factories* 20, 225. <https://doi.org/10.1186/s12934-021-01713-0>
- Clarke, K., Gorley, R., 2006. “PRIMER v6.” User Manual/Tutorial, Plymouth Routine in Multivariate Ecological Research – ScienceOpen.
- Clarke, K.R., 1993. Non-parametric multivariate analyses of changes in community structure. *Australian Journal of Ecology* 18, 117–143. <https://doi.org/10.1111/j.1442-9993.1993.tb00438.x>
- Corre, Y.-M., Bruzaud, S., Audic, J.-L., Grohens, Y., 2012. Morphology and functional properties of commercial polyhydroxyalkanoates: A comprehensive and comparative study. *Polymer Testing* 31, 226–235. <https://doi.org/10.1016/j.polymertesting.2011.11.002>
- Crétois, R., Chenal, J.-M., Sheibat-Othman, N., Monnier, A., Martin, C., Astruz, O., Kurusu, R., Demarquette, N.R., 2016. Physical explanations about the improvement of PolyHydroxyButyrate ductility: Hidden effect of plasticizer on physical ageing. *Polymer, Polymers at Interfaces: Probing Mechanics and Interactions by Atomic Force Microscopy* 102, 176–182. <https://doi.org/10.1016/j.polymer.2016.09.017>
- Deroiné, M., César, G., Le Duigou, A., Davies, P., Bruzaud, S., 2015. Natural Degradation and Biodegradation of Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) in Liquid and Solid Marine Environments. *J Polym Environ* 23, 493–505. <https://doi.org/10.1007/s10924-015-0736-5>
- Deroiné, M., Le Duigou, A., Corre, Y.-M., Le Gac, P.-Y., Davies, P., César, G., Bruzaud, S., 2014. Seawater accelerated ageing of poly(3-hydroxybutyrate-co-3-hydroxyvalerate). *Polymer Degradation and Stability* 105, 237–247. <https://doi.org/10.1016/j.polymdegradstab.2014.04.026>
- Deudero, S., Alomar, C., 2015. Mediterranean marine biodiversity under threat: Reviewing influence of marine litter on species. *Marine Pollution Bulletin* 98, 58–68. <https://doi.org/10.1016/j.marpolbul.2015.07.012>
- Dilkes-Hoffman, L.S., Lant, P.A., Laycock, B., Pratt, S., 2019. The rate of biodegradation of PHA bioplastics in the marine environment: A meta-study. *Marine Pollution Bulletin* 142, 15–24. <https://doi.org/10.1016/j.marpolbul.2019.03.020>

- Dussud, C., Hudec, C., George, M., Fabre, P., Higgs, P., Bruzaud, S., Delort, A.-M., Eyheraguibel, B., Meistertzheim, A.-L., Jacquin, J., Cheng, J., Callac, N., Odobel, C., Rabouille, S., Ghiglione, J.-F., 2018. Colonization of Non-biodegradable and Biodegradable Plastics by Marine Microorganisms. *Frontiers in Microbiology* 9, 1571. <https://doi.org/10.3389/fmicb.2018.01571>
- Fuhrman, J.A., Sleeter, T.D., Carlson, C.A., Proctor, L.M., 1989. Dominance of bacterial biomass in the Sargasso Sea and its ecological implications. *Marine Ecology Progress Series* 57, 207–217.
- Furrer, P., Hany, R., Rentsch, D., Grubelnik, A., Ruth, K., Panke, S., Zinn, M., 2007. Quantitative analysis of bacterial medium-chain-length poly([R]-3-hydroxyalkanoates) by gas chromatography. *Journal of Chromatography A* 1143, 199–206. <https://doi.org/10.1016/j.chroma.2007.01.002>
- Haider, T.P., Völker, C., Kramm, J., Landfester, K., Wurm, F.R., 2019. Plastics of the Future? The Impact of Biodegradable Polymers on the Environment and on Society. *Angewandte Chemie International Edition* 58, 50–62. <https://doi.org/10.1002/anie.201805766>
- Jacquin, J., Cheng, J., Odobel, C., Pandin, C., Conan, P., Pujo-Pay, M., Barbe, V., Meistertzheim, A.-L., Ghiglione, J.-F., 2019. Microbial Ecotoxicology of Marine Plastic Debris: A Review on Colonization and Biodegradation by the “Plastisphere.” *Front. Microbiol.* 10. <https://doi.org/10.3389/fmicb.2019.00865>
- Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R., Law, K.L., 2015. Plastic waste inputs from land into the ocean. *Science* 347, 768–771. <https://doi.org/10.1126/science.1260352>
- Jiang, X., Sun, Z., Ramsay, J.A., Ramsay, B.A., 2013. Fed-batch production of MCL-PHA with elevated 3-hydroxy-nonanoate content. *AMB Express* 3, 50. <https://doi.org/10.1186/2191-0855-3-50>
- Kasuya, K., Mitomo, H., Nakahara, M., Akiba, A., Kudo, T., Doi, Y., 2000. Identification of a Marine Benthic P(3HB)-Degrading Bacterium Isolate and Characterization of Its P(3HB) Depolymerase. *Biomacromolecules* 1, 194–201. <https://doi.org/10.1021/bm9900186>
- Kim, H.M., Ryu, K.E., Bae, K., Rhee, Y.H., 2000. Purification and characterization of extracellular medium-chain-length polyhydroxyalkanoate depolymerase from *Pseudomonas* sp. RY-1. *J. Biosci. Bioeng.* 89, 196–198. [https://doi.org/10.1016/s1389-1723\(00\)88737-x](https://doi.org/10.1016/s1389-1723(00)88737-x)
- Koller, M., Salerno, A., Dias, M.M. de S., Reiterer, A., Brauneegg, G., 2010. Modern Biotechnological Polymer Synthesis: A Review. *Food technology and biotechnology* 48, 255–269.
- Kowalczyk, A., Martin, T.J., Price, O.R., Snape, J.R., van Egmond, R.A., Finnegan, C.J., Schäfer, H., Davenport, R.J., Bending, G.D., 2015. Refinement of biodegradation tests methodologies and the proposed utility of new microbial ecology techniques. *Ecotoxicology and Environmental Safety* 111, 9–22. <https://doi.org/10.1016/j.ecoenv.2014.09.021>
- Kumar, G., Anjana, Hinduja, Sujitha, Dharani, 2020. Review on plastic wastes in marine environment – Biodegradation and biotechnological solutions. *Marine Pollution Bulletin* 150, 110733. <https://doi.org/10.1016/j.marpolbul.2019.110733>
- Laycock, B., Halley, P., Pratt, S., Werker, A., Lant, P., 2014. The chemomechanical properties of microbial polyhydroxyalkanoates. *Progress in Polymer Science, Topical Issue on Biorelated Polymers* 39, 397–442. <https://doi.org/10.1016/j.progpolymsci.2013.06.008>
- Leathers, T.D., Govind, N.S., Greene, R.V., 2000. Biodegradation of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by a Tropical Marine Bacterium, *Pseudoalteromonas* sp. NRRL B-30083. *Journal of Polymers and the Environment* 8, 119–124. <https://doi.org/10.1023/A:1014873731961>
- Lemechko, P., Le Fellic, M., Bruzaud, S., 2019. Production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) using agro-industrial effluents with tunable proportion of 3-hydroxyvalerate monomer units. *International Journal of Biological Macromolecules* 128, 429–434. <https://doi.org/10.1016/j.ijbiomac.2019.01.170>

- López-Ibáñez, S., Beiras, R., 2022. Is a compostable plastic biodegradable in the sea? A rapid standard protocol to test mineralization in marine conditions. *Science of The Total Environment* 831, 154860. <https://doi.org/10.1016/j.scitotenv.2022.154860>
- Maclean, H., Sun, Z., Ramsay, J., Ramsay, B., 2008. Decaying exponential feeding of nonanoic acid for the production of medium-chain-length poly(3-hydroxyalkanoates) by *Pseudomonas putida* KT2440. *Can. J. Chem.* 86, 564–569. <https://doi.org/10.1139/v08-062>
- Martínez-Tobón, D.I., Gul, M., Elias, A.L., Sauvageau, D., 2018. Polyhydroxybutyrate (PHB) biodegradation using bacterial strains with demonstrated and predicted PHB depolymerase activity. *Appl Microbiol Biotechnol* 102, 8049–8067. <https://doi.org/10.1007/s00253-018-9153-8>
- McMurdie, P.J., Holmes, S., 2012. Phyloseq: a bioconductor package for handling and analysis of high-throughput phylogenetic sequence data. *Pac Symp Biocomput* 235–246.
- Meereboer, K.W., Misra, M., Mohanty, A.K., 2020. Review of recent advances in the biodegradability of polyhydroxyalkanoate (PHA) bioplastics and their composites. *Green Chemistry* 22, 5519–5558. <https://doi.org/10.1039/D0GC01647K>
- Moriya, H., Takita, Y., Matsumoto, A., Yamahata, Y., Nishimukai, M., Miyazaki, M., Shimoi, H., Kawai, S.-J., Yamada, M., 2020. Cobetia sp. Bacteria, Which Are Capable of Utilizing Alginate or Waste Laminaria sp. for Poly(3-Hydroxybutyrate) Synthesis, Isolated From a Marine Environment. *Frontiers in Bioengineering and Biotechnology* 8. <https://doi.org/10.3389/fbioe.2020.00974>
- Możejko-Ciesielska, J., Kiewisz, R., 2016. Bacterial polyhydroxyalkanoates: Still fabulous? *Microbiological Research* 192, 271–282. <https://doi.org/10.1016/j.micres.2016.07.010>
- Mukai, K., Yamada, K., Doi, Y., 1993. Enzymatic degradation of poly(hydroxyalkanoates) by a marine bacterium. *Polymer Degradation and Stability* 41, 85–91. [https://doi.org/10.1016/0141-3910\(93\)90066-R](https://doi.org/10.1016/0141-3910(93)90066-R)
- Numata, K., Abe, H., Doi, Y., 2008. Enzymatic processes for biodegradation of poly(hydroxyalkanoate)s crystals. *Canadian Journal of Chemistry* 86, 471–483. <https://doi.org/10.1139/V08-004>
- Numata, K., Abe, H., Iwata, T., 2009. Biodegradability of Poly(hydroxyalkanoate) Materials. *Materials* 2, 1104–1126. <https://doi.org/10.3390/ma2031104>
- Nygaard, D., Yashchuk, O., Hermida, É.B., 2021. PHA granule formation and degradation by *Cupriavidus necator* under different nutritional conditions. *Journal of Basic Microbiology* 61, 825–834. <https://doi.org/10.1002/jobm.202100184>
- Odobel, C., Dussud, C., Philip, L., Derippe, G., Lauters, M., Eyheraguibel, B., Burgaud, G., Ter Halle, A., Meistertzheim, A.-L., Bruzard, S., Barbe, V., Ghiglione, J.-F., 2021. Bacterial Abundance, Diversity and Activity During Long-Term Colonization of Non-biodegradable and Biodegradable Plastics in Seawater. *Frontiers in Microbiology* 12. <https://doi.org/10.3389/fmicb.2021.734782>
- Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M.H.H., Oksanen, M.J., Suggests, M., 2007. The vegan package. *Community ecology package* 10, 719.
- Pachekoski, W.M., Agnelli, J.A.M., Belem, L.P., 2009. Thermal, mechanical and morphological properties of poly (hydroxybutyrate) and polypropylene blends after processing. *Materials Research* 12, 159–164. <https://doi.org/10.1590/S1516-14392009000200008>
- Pachekoski, W.M., Dalmolin, C., Agnelli, J.A.M., 2013. The influence of the industrial processing on the degradation of poly(hydroxybutyrate) - PHB. *Mat. Res.* 16, 237–332. <https://doi.org/10.1590/S1516-14392012005000180>
- Parada, A.E., Needham, D.M., Fuhrman, J.A., 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environmental Microbiology* 18, 1403–1414. <https://doi.org/10.1111/1462-2920.13023>

- Paul-Pont, I., Ghiglione, J.-F., Gastaldi, E., Ter Halle, A., Huvet, A., Bruzaud, S., Lagarde, F., Galgani, F., Duflos, G., George, M., Fabre, P., 2023. Discussion about suitable applications for biodegradable plastics regarding their sources, uses and end of life. *Waste Management* 157, 242–248. <https://doi.org/10.1016/j.wasman.2022.12.022>
- Pérez-Rivero, C., Hernandez-Raquet, G., 2017. Polyhydroxyalkanoates : une alternative ‘bio’ aux plastiques traditionnels. *Innovations Agronomiques* 58, 99–112.
- Prieto, A., Escapa, I.F., Martínez, V., Dinjaski, N., Herencias, C., de la Peña, F., Tarazona, N., Revelles, O., 2016. A holistic view of polyhydroxyalkanoate metabolism in *Pseudomonas putida*. *Environ. Microbiol.* 18, 341–357. <https://doi.org/10.1111/1462-2920.12760>
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 41, D590-596. <https://doi.org/10.1093/nar/gks1219>
- Riis, V., Mai, W., 1988. Gas chromatographic determination of poly- $\beta$ -hydroxybutyric acid in microbial biomass after hydrochloric acid propanolysis. *Journal of Chromatography A* 445, 285–289. [https://doi.org/10.1016/S0021-9673\(01\)84535-0](https://doi.org/10.1016/S0021-9673(01)84535-0)
- Schirmer, A., Jendrossek, D., 1994. Molecular characterization of the extracellular poly(3-hydroxyoctanoic acid) [P(3HO)] depolymerase gene of *Pseudomonas fluorescens* GK13 and of its gene product. *J Bacteriol* 176, 7065–7073.
- Schirmer, A., Jendrossek, D., Schlegel, H.G., 1993. Degradation of poly(3-hydroxyoctanoic acid) [P(3HO)] by bacteria: purification and properties of a P(3HO) depolymerase from *Pseudomonas fluorescens* GK13. *Appl. Environ. Microbiol.* 59, 1220–1227.
- Shen, M., Huang, W., Chen, M., Song, B., Zeng, G., Zhang, Y., 2020. (Micro)plastic crisis: Unignorable contribution to global greenhouse gas emissions and climate change. *Journal of Cleaner Production* 254, 120138. <https://doi.org/10.1016/j.jclepro.2020.120138>
- Simon, M., Azam, F., 1989. Protein content and protein synthesis rates of planktonic marine bacteria. *Mar. Ecol. Prog. Ser.* 51, 201–213. <https://doi.org/10.3354/meps051201>
- Sun, Z., Ramsay, J.A., Guay, M., Ramsay, B.A., 2006. Automated feeding strategies for high-cell-density fed-batch cultivation of *Pseudomonas putida* KT2440. *Appl Microbiol Biotechnol* 71, 423–431. <https://doi.org/10.1007/s00253-005-0191-7>
- Thomas, T., Elain, A., Bazire, A., Bruzaud, S., 2019. Complete genome sequence of the halophilic PHA-producing bacterium *Halomonas* sp. SF2003: insights into its biotechnological potential. *World J Microbiol Biotechnol* 35, 50. <https://doi.org/10.1007/s11274-019-2627-8>
- Volant, C., Balnois, E., Vignaud, G., Magueresse, A., Bruzaud, S., 2022. Design of Polyhydroxyalkanoate (PHA) Microbeads with Tunable Functional Properties and High Biodegradability in Seawater. *J Polym Environ* 30, 2254–2269. <https://doi.org/10.1007/s10924-021-02345-6>
- Volova, T.G., Boyandin, A.N., Vasil'ev, A.D., Karpov, V.A., Kozhevnikov, I.V., Prudnikova, S.V., Rudnev, V.P., Xuân, B.B., Dũng, V.V., Gitel'zon, I.I., 2011. Biodegradation of polyhydroxyalkanoates (PHAs) in the South China Sea and identification of PHA-degrading bacteria. *Microbiology* 80, 252–260. <https://doi.org/10.1134/S0026261711020184>
- Volova, T.G., Boyandin, A.N., Vasiliev, A.D., Karpov, V.A., Prudnikova, S.V., Mishukova, O.V., Boyarskikh, U.A., Filipenko, M.L., Rudnev, V.P., Bá Xuân, B., Việt Dũng, V., Gitelson, I.I., 2010. Biodegradation of polyhydroxyalkanoates (PHAs) in tropical coastal waters and identification of PHA-degrading bacteria. *Polymer Degradation and Stability* 95, 2350–2359. <https://doi.org/10.1016/j.polymdegradstab.2010.08.023>
- Wickham, H., 2016. *ggplot2: elegant graphics for data analysis*. Springer-Verlag New York, 2016. Springer-Verlag New York.
- Wright, R.J., Erni-Cassola, G., Zadjelovic, V., Latva, M., Christie-Oleza, J.A., 2020. Marine Plastic Debris: A New Surface for Microbial Colonization. *Environ Sci Technol* 54, 11657–11672. <https://doi.org/10.1021/acs.est.0c02305>

- Xie, Y., Noda, I., Akpalu, Y.A., 2008. Influence of cooling rate on the thermal behavior and solid-state morphologies of polyhydroxyalkanoates. *Journal of Applied Polymer Science* 109, 2259–2268. <https://doi.org/10.1002/app.28278>
- Young, K.D., Chul, K.H., Young, K.S., Ha, R.Y., 2005. Molecular Characterization of Extracellular Medium-chain-length Poly(3-hydroxyalkanoate) Depolymerase Genes from *Pseudomonas alcaligenes* Strains. *Journal of Microbiology* 43, 285–294.
- Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A., 2013. Life in the “Plastisphere”: Microbial Communities on Plastic Marine Debris. *Environ. Sci. Technol.* 47, 7137–7146. <https://doi.org/10.1021/es401288x>

## Appendice A

Minimal medium composition : : NaCl 24 g.L<sup>-1</sup>, Na<sub>2</sub>SO<sub>4</sub> 4 g.L<sup>-1</sup>, KCl 0.68 g.L<sup>-1</sup>, KBr 0.1 g.L<sup>-1</sup>, H<sub>3</sub>BO<sub>3</sub> 0.025 g.L<sup>-1</sup>, NaF 0.002 g.L<sup>-1</sup>, MgCl<sub>2</sub>·6H<sub>2</sub>O 10.8 g.L<sup>-1</sup>, CaCl<sub>2</sub>·2H<sub>2</sub>O 1.5 g.L<sup>-1</sup>, SrCl<sub>2</sub>·6H<sub>2</sub>O 0.024 g.L<sup>-1</sup>, NaHCO<sub>3</sub> 0.2 g.L<sup>-1</sup>, NaHPO<sub>4</sub> 0.04 g.L<sup>-1</sup>, NH<sub>4</sub>Cl 0.5 g.L<sup>-1</sup>, FeCl<sub>3</sub> 4 g.L<sup>-1</sup>, EDTA 2 g.L<sup>-1</sup>, 1 mL of traces elements for 1 L of medium composed of: CuCl<sub>2</sub>·2H<sub>2</sub>O 0.015 g.L<sup>-1</sup>, NiCl<sub>2</sub>·H<sub>2</sub>O 0.025 g.L<sup>-1</sup>, Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O 0.025 g.L<sup>-1</sup>, ZnCl<sub>2</sub> 0.07 g.L<sup>-1</sup>, MnCl<sub>2</sub>·4H<sub>2</sub>O 0.1 g.L<sup>-1</sup>, CoCl<sub>2</sub>·6H<sub>2</sub>O 0.12 g.L<sup>-1</sup> and 1 mL of a vitamin solution for 1 L of medium composed of: p-aminobenzoic acid 0.005 g.L<sup>-1</sup>, pyridoxine-HCl 0.1 g.L<sup>-1</sup>, thiamine-HCl 0.05 g.L<sup>-1</sup>, riboflavin 0.05 g.L<sup>-1</sup>, nicotinic acide 0.05 g.L<sup>-1</sup>, D-Ca-pantothenate 5.10<sup>-9</sup> g.L<sup>-1</sup>, lipoic acid 0.05 g.L<sup>-1</sup>, nicotinamide 0.05 g.L<sup>-1</sup>, B12 vitamin 0.05 g.L<sup>-1</sup>, biotine 0.02 g.L<sup>-1</sup> and folic acid 0.02 g.L<sup>-1</sup>.



# Chapitre 3 : Dégradation enzymatique de polyhydroxyalkanoates à chaînes courtes (scl-PHA) par une nouvelle PHA depolymérase isolée de la souche marine *Alteromonas plasticoclasticus* MED1



## Préambule

Ce chapitre est écrit sous forme d'un article scientifique en anglais qui sera soumis prochainement au journal *Applied Microbiology and Biotechnology*. Les résultats présentés proviennent d'une collaboration multidisciplinaire entre 4 laboratoires : l'IRD, le LOMIC, le Genoscope et l'ICCF. Ce travail fait suite à la découverte d'une nouvelle souche *Alteromonas plasticoclasticus* *MEDI* isolée à partir de la plastisphère de PHBHV en milieu marin pour ses capacités de biodégradation de ce polymère (Barbe et al., Annexe F). L'analyse de son génome a révélé la présence de gènes codant pour une PHA dépolymérase (dPhaZ<sub>SCL</sub>). Cet article fait également suite à l'étude de la biodégradation des PHA en milieu naturel marin du chapitre 2.

Les objectifs de cette étude sont:

- (1) Caractériser et étudier la nouvelle enzyme dPhaZ<sub>SCL</sub> d'*Alteromonas plasticoclasticus* *MEDI*,
- (2) Comparer son efficacité par rapport à deux autres dPhaZ<sub>SCL</sub> bien étudiées provenant de la littérature (dPhaZ<sub>SCL</sub> de *Ralstonia pickettii* T1 et d'*Alcaligenes faecalis* AE122) et
- (3) Etudier l'influence des propriétés physico-chimiques de deux *scl*-PHA différents (PHB et de PHBHV11) sur leur dégradation *in vitro* par voie enzymatique.

Les trois enzymes ont pu être produites à partir des trois souches bactériennes ciblées par des techniques de biologie moléculaire et de biochimie. Les réactions de dégradation enzymatique ont été réalisées à partir de ces enzymes sur du PHBHV3 en poudre et des films de PHB et de PHBHV11 durant 7h ou 40h. La dégradation de PHBHV3 a été évaluée par suivi de la diminution de la turbidité (densité optique à 650 nm) pendant que celles des films de PHB et PHBHV11 ont été suivies par microscopie électronique à balayage, perte de poids, dosage et identifications des oligomères et monomères de PHA produits et solubles.

L'activité de la dPhaZ<sub>SCL</sub> d'*Alteromonas plasticoclasticus* *MEDI* a été confirmée autant sur la poudre de PHBHV que sur les films de *scl*-PHA. Son activité est plus importante sur le film de PHBHV que sur le film de PHB, suggérant un impact des régions amorphes sur l'efficacité de l'enzyme.

Comparée aux deux autres enzymes déjà caractérisées, son activité est cependant plus faible dans nos conditions expérimentales, ce qui a conduit à la détection d'une plus grande diversité de motifs d'oligomères solubles. Les conditions réactionnelles utilisées dans cette étude telles que la température, le pH, le tampon de réaction ou encore l'étape de purification de dPhaZ-Apla restent à

approfondir pour tenter d'améliorer la cinétique de transformation du substrat par cette nouvelle enzyme.

# Production and characterization of a novel extracellular short chain-length polyhydroxyalkanoate depolymerase from the marine *Alteromonas plasticoclasticus* MED1

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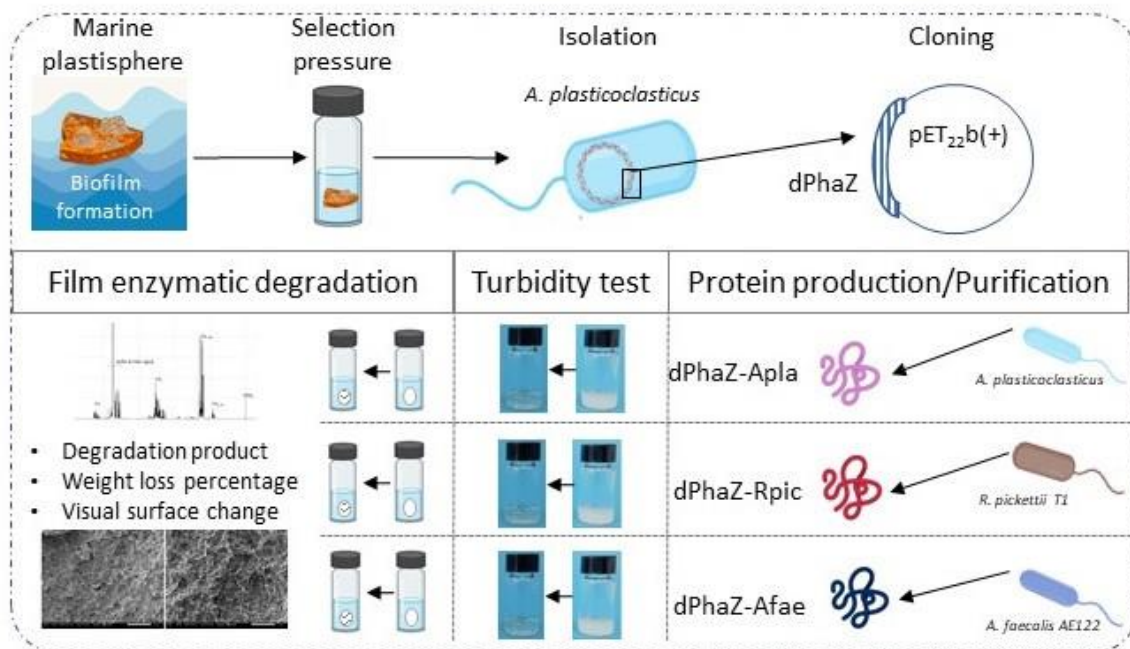
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**Keywords :** Biodegradable polymer, PHA depolymerase, oligomers

## Highlights :

- Production of *scl*-PHA depolymerase (dPhaZ-Apla) by a marine bacterium
- dPhaZ-Apla depolymerase is more efficient on PHBHV11 than on PHB
- dPhaZ-Apla showed lower activity as compared to other known depolymerases, thus conducting to more diverse oligomers release

## Graphical abstract



## Abstract

Polyhydroxyalkanoates (PHA) are biodegradable polymers synthesized by a wide range of microorganisms. Among the PHA family, short chain length-PHA showed great degradation capacities in the environment. A recent study pointed out the biodegradation capabilities of poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBHV) by the novel marine bacteria *Alteromonas plasticoclasticus* MED1 isolated from the marine plastisphere. In this study, the extracellular PHA depolymerase dPhaZ-Apla from *A. plasticoclasticus* MED1 was produced and purified by cloning of competent cells. Enzymatic activities were measured by following the decrease in turbidity on PHBHV powder, as well as by following the visual changes of surface, the quantity and composition of the released oligomers (Nuclear Magnetic Resonance spectroscopy, NMR and mass spectrometry LC / HRMS) and the weight loss percentage on PHB and PHBHV films. The dPhaZ-Apla presented a catalytic site of type I and two substrate-binding domains (SBD). In our experimental conditions, it presented lower degradation rates than two other dPhaZ<sub>SCL</sub> enzymes (dPhaZ-Rpic from *Ralstonia pickettii* T1 and dPhaZ-Afae from *Alcaligenes faecalis* AE122). Finally, dPhaZ-Apla depolymerase showed a higher degradation of the more amorphous PHBHV than the more crystalline PHB.

## 1. Introduction

Polyhydroxyalkanoate (PHA) is a bio-sourced and biodegradable polyester polymer with properties resembling to common non degradable petroleum-based polymers (Sudesh et al., 2000). Gradual replacement of non-degradable plastics with eco-friendly biodegradable materials is one of the solutions to prevent non-biodegradable plastic accumulation in the environment for selected applications with respect to their use and end of life (Paul-Pont et al., 2023). From bacterial origin, PHA serve as carbon and energy reserve materials and according to the strain and the carbon source used, they display a wide range of chemical compositions and properties (Laycock et al., 2014). Poly (3-hydroxybutyrate) (PHB) homopolymer is the most commonly occurring PHA in the natural environment, hence the most commonly used in PHA related studies (Khosravi-Darani et al., 2013). With high crystallinity (>50%), it is a relatively brittle and stiff polymer (Koller et al., 2010). Production of its copolymer with valeric acid triggers the incorporation of 3-hydroxyvalerate (HV) and results in a less stiff and brittle polymer easier to process for commercial applications (Pachekoski et al., 2009).

Plastic biodegradability depends on the microorganisms living on plastics, i.e. the so-called 'plastisphere' (Zettler et al., 2013). Recent studies showed that specific marine microbial diversity was selected on PHA as compared to conventional polyolefins films (polyethylene or polystyrene), which differed from the surrounding seawater (Odobel et al. 2021). Bacteria have been isolated from various environments for their capability to utilize extracellular PHA as a sole carbon source and energy, including *Ralstonia picketti T1* (Tanio et al., 1982), *Alcaligenes faecalis AE122* (Kita et al., 1997), *Comamonas* sp. (Mukai et al., 1993a), *Acidovorax* sp. TP4 (Wang et al., 2002) and *Streptomyces* sp. SFB5A (Blevins et al., 2018).

The ability of microorganisms to degrade PHA in the environment is mainly dependent on the secretion of specific extracellular PHA depolymerases (Brandi et al., 1995). PHA depolymerases are highly specific with respect to the length of the monomer carbon side chain of the PHA substrate, with distinct enzymes involved in the short-chain-length (*scl*-; 3 to 5 carbon atoms) versus medium-chain-length PHA (*mcl*-; 6 to 14 carbon atoms). PHA depolymerases degrading *scl*-PHA (dPhaZ<sub>SCL</sub>) are the most studied since *mcl*-PHA are not produced in industrial quantities. A dedicated database for PHA depolymerase has been established (DED, <http://www.ded.uni-stuttgart.de>; Knoll et al., 2009) to inventory dPhaZ. All dPhaZ<sub>SCL</sub> depolymerase proteins have a composite domain structure made of a signal peptide, a large catalytic domain (N-terminal), a linking domain and a substrate-binding domain (SBD; C-terminal). These enzymes are secreted into the surrounding medium and

hydrolyze PHA into their oligomers or monomers allowing the smaller molecules to cross the bacterial membrane for further assimilation (Jendrossek et al., 1996).

Advances in molecular biology, high-throughput ‘omic’ techniques and computational tools allow to combine *in silico* genome scale metabolic network analysis with bacterial physiology and metabolism to build a holistic standpoint. In this study, we used the genome analysis of a marine bacterium recently isolated from the marine plastisphere on PHA films to describe a new dPhaZ<sub>SCL</sub> depolymerase. The *phaZ* gene of *Alteromonas plasticoclasticus* MED1 has been cloned and amplified in order to investigate the amino acid sequence and the associated enzymatic activity of the purified dPhaZ<sub>SCL</sub> depolymerase. We hypothesised that this enzyme had similar activities on two different scl-PHA (PHB and PHBV) when compared to two other known extracellular dPhaZ<sub>SCL</sub> from *Ralstonia pickettii* T1 and *Alcaligenes faecalis* AE122 by using turbidity tests, weight loss and scanning electron microscopy. We also tested the oligomers and monomers releases (by using Nuclear Magnetic Resonance spectroscopy) in order to compare the degradation products of the three dPhaZ<sub>SCL</sub> depolymerases.

## 2. Material and methods

### 2.1. Production of dPhaZ<sub>SCL</sub> depolymerase from three bacterial strains

The gene originated from *Alteromonas plasticoclasticus* MED1 isolated from a natural biofilm growing on PHBV in seawater (Barbe et al., submitted, Annexe F) was compared to two other *phaZ* genes from *Ralstonia pickettii* T1 (Shirakura et al., 1986) and *Alcaligenes faecalis* AE122 (Kita et al., 1997). The corresponding dPhaZ<sub>SCL</sub> depolymerases are called “dPhaZ-Apla”, “dPhaZ-Rpic” and “dPhaZ-Afae” hereafter for *A. plasticoclasticus* MED1, *Ralstonia pickettii* T1 (P94146, database UniProtKB, <https://www.uniprot.org>) and *Alcaligenes faecalis* AE122 (P12625), respectively. The 3 *phaZ* genes were synthesized by Twist Bioscience (San Francisco, United States) and the same protocol was applied as followed. Genes were amplified by addition of specific extensions for cloning into pET22b(+) (Novagen) modified for ligation independent cloning (LIC) to the primers. The reverse primers introduced a hexahistidine tag sequence in the C-terminal of the proteins for further purification. The plasmids were transformed into BL21-CodonPlus (DE3)-RIPL competent cells (Agilent technologies).

After growing transformed cells of *R. pickettii* T1 and *A. faecalis* AE122 over-night at 37°C, 250 ml of Terrific Broth medium containing 500 mM Sorbitol, 5 mM betaine and 100 µg/ml carbenicillin were inoculated and kept growing to an O.D. of 1.8 at 37°C. Isopropyl β-D-thiogalactopyranoside was added at a concentration of 0.5 mM to induce protein production, and the

cells were further grown at 20 °C overnight. The cell lysis was performed in a phosphate buffer (50 mM Phosphate (Na/K), 500 mM NaCl, 30 mM imidazole, 15% glycérol, pH 8,0) with BugBuster® protein extraction reagent. The proteins were purified by nickel chromatogry (His Trap FF 5ml ) in tandem with gel filtration (Hi load 16/600 superdex), as described previously (Perchat et al., 2018). The final buffer was 50 mM NaCl, 50mM Tris pH7,5, glycerol 15%. The protein ALSP-2657 isolated from *Alteromonas plasticoclasticus MED1* was produced from a culture of 50 mL at 37°C and lysed in a Tris-HCl buffer (50 mM NaCl, 50mM Tris pH7,5, glycerol 10%, 30 mM imidazole, 1 mM pefabloc and lysonase® (Novagen) by sonication and purified by QIAexpress Ni-NTA Protein Purification System (Qiagen) following the manufacturer's instructions. Elution buffer was 50 mM NaCl, 50mM Tris pH7.5, glycerol 10%, 250 mM imidazole. Protein concentrations were determined by the Bradford method with bovine serum albumin as standard. The samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (NuPAGE system – Invitrogen). The purified proteins were stored at - 80°C.

## 2.2. Alignment and comparison of the amino acids sequences of the dPhaZ depolymerases

Amino acid sequence analysis and comparison were realised from the originated gene sequences on ClustalOmega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>).

## 2.3. Enzymatic assay based on turbidity tests

A first set of enzymatic degradation assay was carried out using the commercial PHBHV Y1000P in the form of powder (composed of 97% HB and 3% HV, Tianan Biological Materials, China). Tests were performed at 37 °C in 100 mM potassium phosphate buffer (pH 7.5) for dPhaZ-Rpic and dPhaZ-Afae and 50 mM Tris-HCl buffer, 1 mM CaCl<sub>2</sub> (pH 7.5) for dPhaZ-Apla with gentle shaking (120 rpm) for 7h. A stable suspension of PHBHV Y1000P (Tianan, China) in respective buffers was obtained by a 3 cycle treatment of 1 min vortex and 3 min in an ultrasonic bath (40 kHz, Branson Ultrasonic bath 3800, Connecticut, USA). Activities of dPhaZ enzymes were assayed by the decrease in turbidity with PHBHV according to the method of Tanio et al. (1982). The standard reaction mixture contained the respective buffer, 1 µg of the respective enzyme dPhaZ-Rpic, dPhaZ-Afae or dPhaZ-Apla and 5 mg of PHBHV Y1000P powder in a final volume of 1 mL. The reaction started with the addition of the enzyme. Optical density at 650 nm was measured on duplicate samples at 0, 2, 4.5 and 7 hours with a spectrophotometer (Ultrospec 10, Biochrom, UK). Blanks were realised using the same reaction mixture but without the enzyme or without the PHBHV powder.

## 2.4. Enzymatic assays to measure changes in surface properties (SEM) and degradation products (<sup>1</sup>H NMR, LC / HRMS)

Films of PHB and PHBHV11 were prepared by slow solvent casting. Briefly, 0.5 g of PHA were dissolved in chloroform and poured in glass Petri dishes with their lids and put under a fume hood. The lids were displaced twice a day to allow renewal of the air within. All films were solvent-casted on the same day and were allowed to age for 3 days at room temperature. Melting enthalpy measurements witnessing the crystallinity were measured as previously described (Lemechko et al., 2019) and showed a  $\Delta H_m$  of 87 and 40 J.g<sup>-1</sup> for PHB (100% HB) and PHBHV11 (89% HB and 11% HV), respectively. Disc of polyester films of 56.5 mm<sup>2</sup> total surface each were prepared for further enzymatic degradation tests that were carried out at 37 °C with gentle shaking (120 rpm) for 40h. Standard reaction mixture contained a disc of PHA, 100 mM buffer (pH 7.5) for dPhaZ-Rpic and dPhaZ-Afae, 50 mM Tris-HCl buffer, 1 mM CaCl<sub>2</sub> (pH 7.5) for dPhaZ-Apla and 1 µg of the respective enzyme. Sampling were taken in triplicate at 0, 8, 16, 24, 32 and 40 hours. For all analysis, discs of PHA were removed, washed with distilled water, dried to constant weight using a freeze dryer and kept at room temperature in the dark until further analysis. Blanks were realized following the same protocol but without enzyme addition.

Surface film degradation was followed by scanning electron microscope (JSM-IT500HR from Jeol) as previously described by (Kedzierski et al., 2018). SEM observations were carried out with secondary electron detector at an acceleration voltage of 3 kV. Discs that underwent enzymatic degradation with dPhaZ-Apla, dPhaZ-Rpic or dPhaZ-Afae during 32 hours (as described above) were stuck on an adhesive carbon tape and gold coated with a sputter coater (Scancoat6 from Edwards).

Weight loss measurements were measured on a precision balance from 3 discs that underwent the enzymatic attack or not (control) at 40h and were averaged. Results are expressed in percentage of weight loss compared to 0 hour.

Enzymatic degradation products were analysed from the supernatants of the enzymatic assays on samples by Proton Nuclear Magnetic Resonance spectroscopy (<sup>1</sup>H NMR), as previously described (Eyheraguibel et al., 2017). Briefly, one milliliter was filtrated using pre-rinsed amicon ultra 0.5 (30kd) centrifugal filter unit to remove residual proteins and then lyophilized. The dry samples were resuspended in 600µl of 300mM phosphate buffer (pH 2.6) in Deuterated water (D<sub>2</sub>O, Eurisotop), and 0.2 mM of deuterated trimethylsilylpropanoic acid sodium salt (TSPd<sub>4</sub>, Eurisotop). D<sub>2</sub>O was used for locking and shimming while TSPd<sub>4</sub> constituted a reference for chemical shifts (<sup>1</sup>H,  $\delta$  0.00 ppm) and quantification. <sup>1</sup>H NMR spectra were recorded on a *Bruker* Avance 500 MHz spectrometer,



equipped with a 5 mm inverse-triple tuned (TXI)  $^1\text{H}/^{13}\text{C}/^{15}\text{N}$  with z-gradient coil probe (Bruker Biospin Wissenbourg, France), with 5 mm-diameter tubes containing 600  $\mu\text{l}$  of sample. The spectral peaks were assigned by comparing chemical shift and multiplicity with 3-hydroxybutyric acid and 3-hydroxyvaleric acid standards and the literature. Discs of PHA without addition of enzymes were also sampled and analyzed as negative controls. To compare the samples, the NMR signals were calibrated according to the internal deuterated standard TSPd<sub>4</sub> and the whole spectrum signal was integrated to determine the relative quantity of degradation products (oligomers) in the samples.

The monomer compositions were analyzed with an Ultimate 3000 RSLC chromatographic system (ThermoScientific) coupled to an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray (ESI) source. The chromatographic separations were performed using a Kinetex EVO C18 column (2,1 x 100 mm; 1,7 $\mu\text{m}$ -Phenomenex) operating at 30°C and an injection volume of 5 $\mu\text{l}$ . The flow rate was fixed at 0.45 mL/min with 0.1% of formic acid in water and 0.1% of formic acid in acetonitrile for mobile phases at the following gradient: initial, 95% A; 0–7.5 min linear, 1% A linear; 7.5–8.5 min, 1% A; 8.5–9 min, 95% A linear, 9-11 min 95% A following by washing and reconditioning of the column. The mass spectrometer was operated in positive ion mode with capillary voltage at 3,2 kV and a capillary temperature set at 320 °C. The detection was performed with full scan from m/z 50 to 750 using a resolution set at 70 000 at m/z 200. HRMS raw data were processed with Xcalibur Software (version 4.1) considering the m/z values with a peak intensity strictly greater than 10<sup>4</sup>.

## 2.5. Statistical analysis

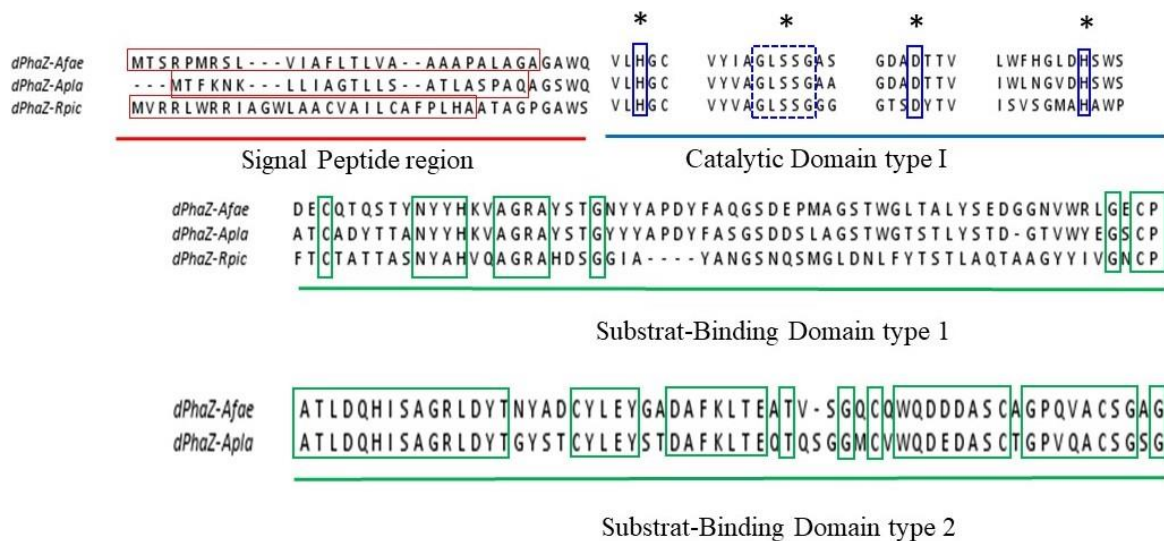
Statistical analysis was performed on R studio (R Core Team, 2022, version 4.2.2). Amounts of 3-hydroxybutyrate (HB) units released in the supernatants at 40h underwent a Student's t-test to highlight any differences between the polymer composition for each enzyme whereas a ANOVA was performed to determine the differences between the three dPhaZ-Apla, dPhaZ-Rpic and dPhaZ-Afae enzymes on each polymer.

## 3. Results

### 3.1. Comparison of the amino acid sequence of dPhaZ-Apla with dPhaZ-Rpic and dPhaZ-Afae

Alignment of amino acid sequences of the dPhaZ-Apla revealed a percentage homology of 31.4 % and 64.7 % with dPhaZ-Rpic and dPhaZ-Afae, respectively. Signal peptide sequences were very variable while dPhaZ-Apla exhibited an amino acid sequence corresponding to a catalytic domain of

type I similar to dPhaZ-Rpic and dPhaZ-Afae which contains the catalytic triad pattern Histidine – Aspartic acid – Histidine and the lipase box between the first histidine and the aspartic acid (Fig. 1). Similar degrees of homology were found on this catalytic domain amino acid region when compared dPhaZ-Apla to dPhaZ-Afae (69.2%) and to dPhaZ-Rpic (32.1). Analysis of the Substrate Binding Domain (SBD) sequence of dPhaZ-Apla revealed a SBD type I with a homology of 28.8% and 67.2 compared to dPhaZ-Rpic and dPhaZ-Afae, respectively. A second SBD of type II was identified on dPhaZ-Apla and similar to dPhaZ-Afae (78.3% of homology). No SBD type II was identified in dPhaZ-Rpic (Fig. 1).



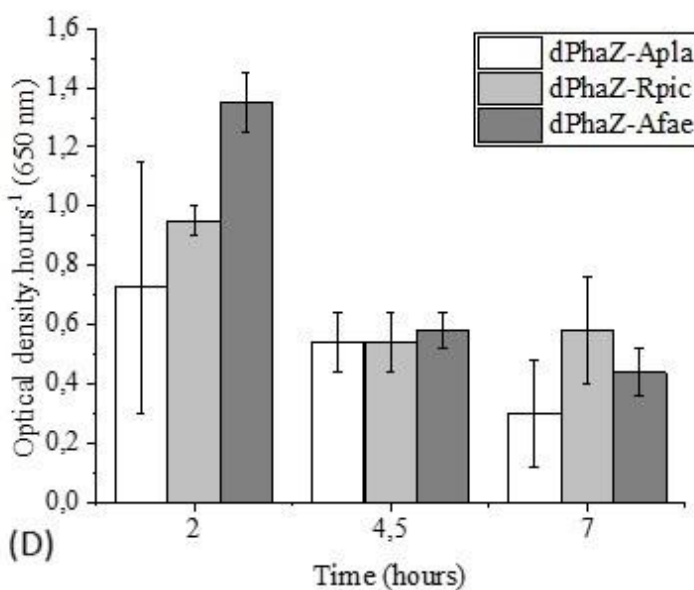
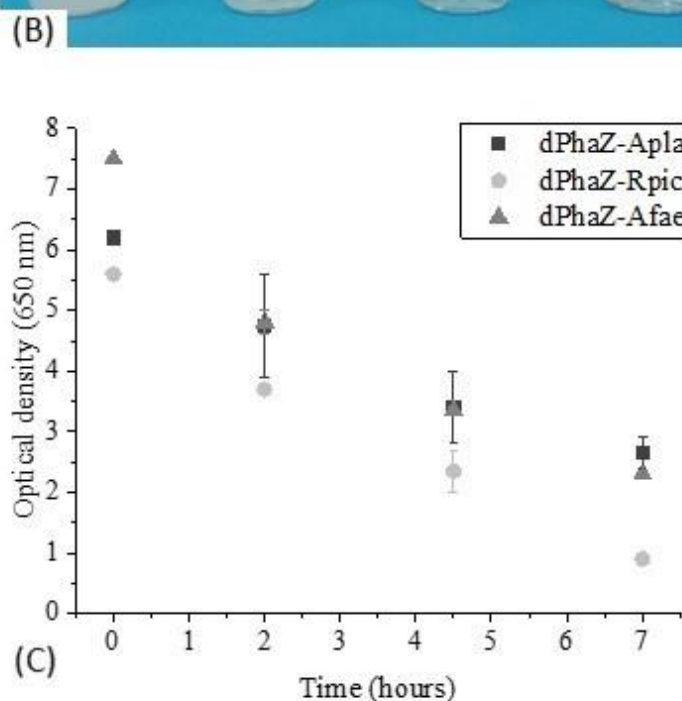
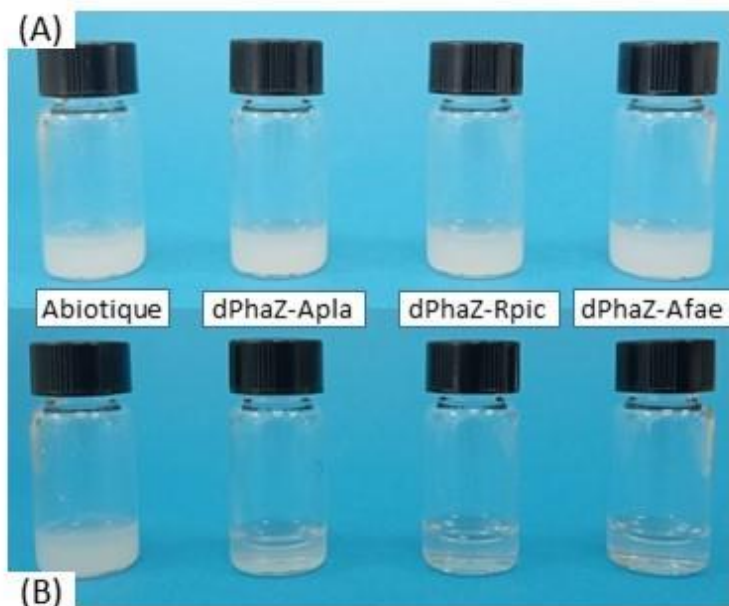
**Figure 1:** Deduced amino acid sequences of the dPhaZ depolymerases of the signal peptide region, the catalytic domain type I and the two SBD. (\*) Amino acids composing the catalytic triad and the lipase box are blue-boxed. Identical amino acids matching the same positions of the SBD are green-boxed.

### 3.2. Comparison between dPhaZ-Apla, dPhaZ-Rpic and dPhaZ-Afae depolymerase activities by turbidity tests

Firstly, decrease in turbidity was clearly noticeable after 7h for the three enzymes (Fig. 1A and 1B) leading to translucent solutions when compared to the abiotic control (no enzyme addition). The transformation of PHBHV powder into water-soluble compounds was measured by following the optical density at 650 nm. Activity of dPhaZ-Apla followed the same kinetic as for the well-known dPhaZ-Rpic and dPhaZ-Afae enzymes, especially during the first 4.5 hours (Fig. 1C). After 4.5 hours, activities of dPhaZ-Apla et dPhaZ-Afae showed a lower final turbidity ( $2.65 \pm 0.25$  and  $2.3 \pm 0.03$  OD, respectively) than dPhaZ-Rpic ( $0.90 \pm 0.01$  OD) (Fig. 1C). Degradation rate of dPhaZ-Apla tended to decrease over time ( $0.73 \pm 0.43$  OD.h<sup>-1</sup> at 2 hours then  $0.54 \pm 0.1$  OD.h<sup>-1</sup> and  $0.3 \pm 0.18$  OD.h<sup>-1</sup> at 4.5 and 7 hours) (Fig. 1D). Overall, activities diminished over time for all enzymes, with

higher decrease observed for dPhaZ-Apla at the end of the experiment compared to dPhaZ-Rpic and

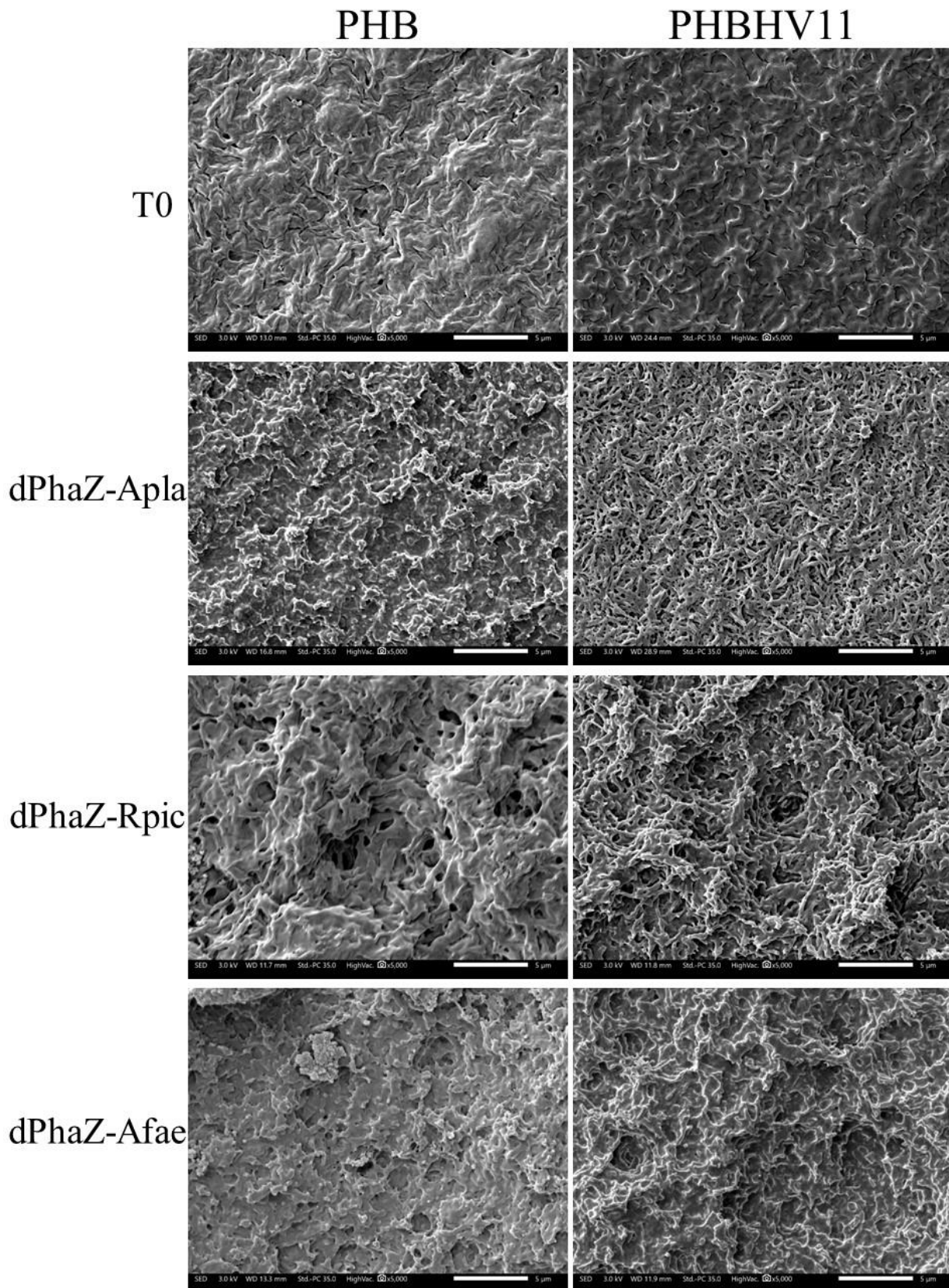
dPhaZ-Afae activities that remained stable from 4.5 to 7 hours ( $0.58 \pm 0.18$  and  $0.44 \pm 0.08$  OD.h<sup>-1</sup> at 7 h for dPhaZ-Rpic and dPhaZ-Apla, respectively) (Fig. 1D). Negative controls that consisted in PHBHV powder in buffers without addition of enzyme did not show any change in turbidity over time.



**Figure 2:** PHBHV-degrading activity of the dPhaZ depolymerases. (A): Visual turbidity of solutions composed of 1 µg of each enzyme (dPhaZ-Apla, dPhaZ-Rpic and dPhaZ-Afae) in 1 mL of buffer solutions containing 5 mg.mL<sup>-1</sup> of PHBHV powder at 0 hour. (B) Visual turbidity of solutions composed of 1 µg of each enzyme (dPhaZ-Apla, dPhaZ-Rpic and dPhaZ-Afae) in 1 mL of buffer solutions containing 5 mg.mL<sup>-1</sup> of PHBHV powder after 40 h at 37°C and 120 rpm. (C): Optical density (650 nm) 0, 2, 4.5 and 7 hours and (D): Speed of decrease in turbidity (optical density per hours) at 2, 4.5 and 7 hours

### 3.3. Visual surface film degradation by dPhaZ-Apla, dPhaZ-Rpic and dPhaZ-Afae depolymerases observed by scanning electron microscopy

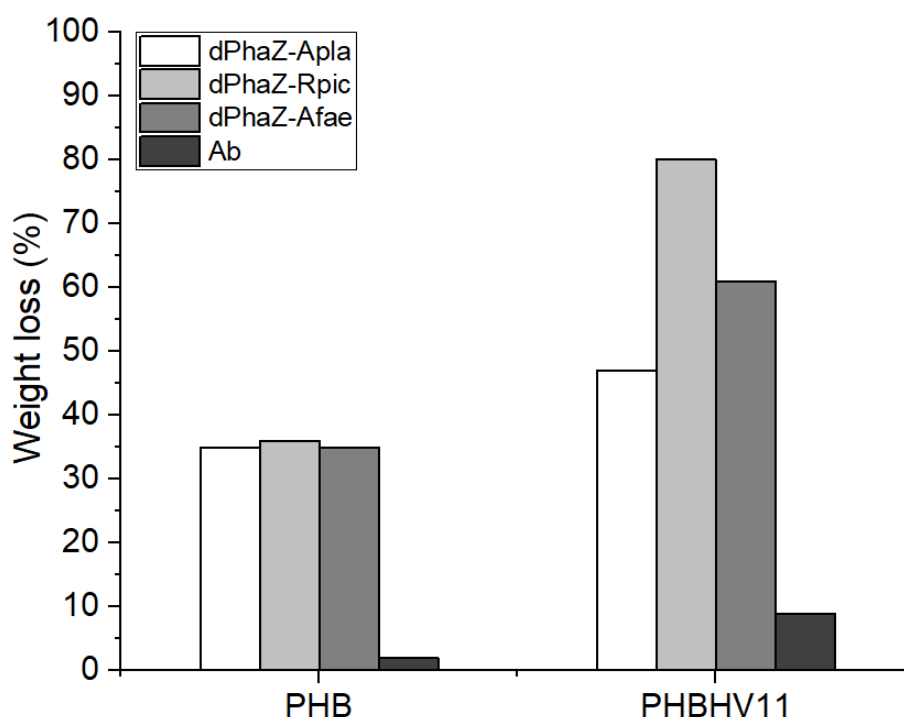
Scanning electron microscopy of the three enzymatic attack after 32 hours showed visual surface changes for both *scl*-PHA (PHB and PHBHV11). Enzymatic degradation of dPhaZ-Apla showed a surface erosion of smooth regions leading to the formation of a more porous-type and more fiber-structured material compared to T0 especially for PHBHV11, while some craters surrounded of structured regions are visible on PHB. Enzymatic degradation of dPhaZ-Rpic on the same polymers showed the same trends with a more pronounced effect. Craters on PHB were deeper and larger. Reliefs of the structured regions were more visible on PHBHV11 on dPhaZ-Rpic attack compared to dPhaZ-Apla enzymatic degradation. The same phenomenon was observed for dPhaZ-Afae enzymatic degradation on PHBHV11, while PHB degradation with this latter enzyme showed a smooth erosion with the disappearance of large structured regions that have been detached for the surface of the materials.



**Figure 3:** SEM images of PHB and PHBHV11 surfaces after 32h or 0 hours (T0, blank) of enzymatic degradation by dPhaZ-Apla, dPhaZ-Rpic and dPhaZ-Afae. White rectangle at the right bottom is 5  $\mu\text{m}$  scale.

### 3.4. Weight loss percentage of PHB and PHBHV films after enzymatic attack by dPhaZ-Apla, dPhaZ-Rpic and dPhaZ-Afae

Weight loss percentage of PHB at 40 h reached 35, 36 and 35% for dPhaZ-Apla, dPhaZ-Rpic and dPhaZ\_Afae, respectively (Figure 3). Maximal weight loss was obtained on PHBHV11, reaching 61% and 80% from dPhaZ-Rpic and dPhaZ-Afae, respectively. Weight loss percentage of dPhaZ-Apla was lower on this polymer and reached 47%.



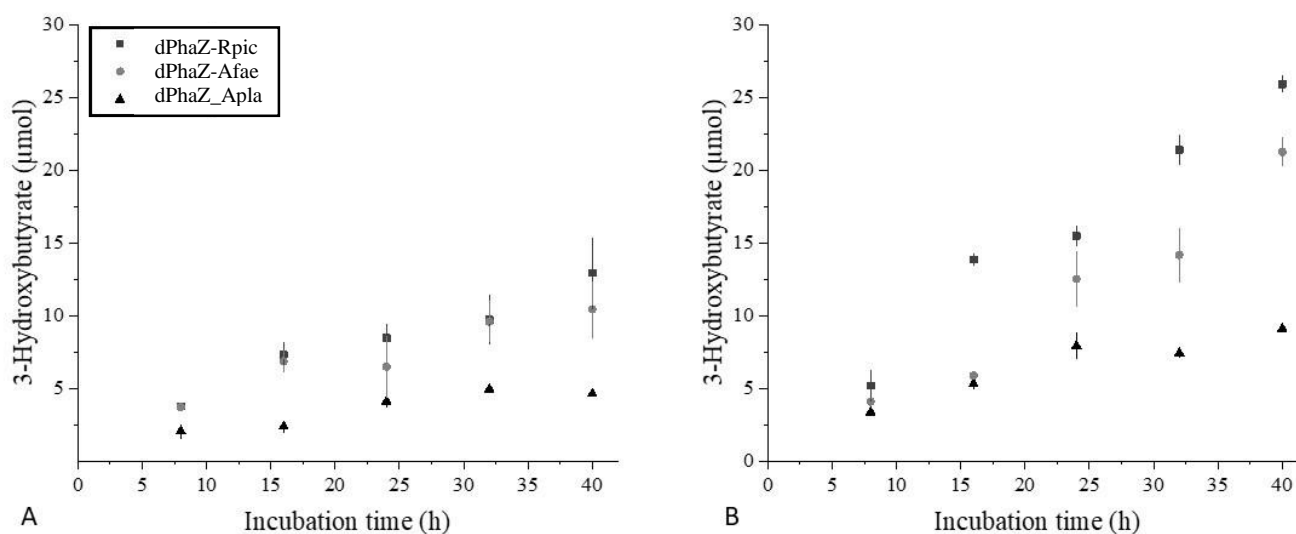
**Figure 4:** Weight loss percentage of PHB and PHBHV11 at 40 h of enzymatic attack from dPhaZ-Apla, dPhaZ-Rpic and dPhaZ-Afae

### 3.5. Characterization of the degradation products by <sup>1</sup>H NMR

The release of PHB and PHBHV11 oligomers was quantified by <sup>1</sup>H NMR. Fig. 1S (Supplemental data) shows the <sup>1</sup>H NMR spectra of PHB (A) and PHBHV (B) soluble oligomers. The peak assignment of the PHB and PHBHV NMR spectra showed that the chemical shift of protons from CH<sub>2</sub> ( $\delta$  2.2-2.8 ppm) and CH ( $\delta$  4.2-5.2 ppm) were the same for hydroxybutyrate (HB) and hydroxyvalerate units (HV). However, it was possible to distinguish clearly the chemical shift of the protons from the methyl group of hydroxybutyrate (CH<sub>3</sub>-HB  $\delta$  1.2 ppm) and hydroxyvalerate (CH<sub>3</sub>-HV  $\delta$  0.8 ppm). These specific signals were used to quantify and compare the number of micromoles of hydroxybutyrate and hydroxyvalerate units produced by the different enzymes on PHBHV (Table 1S,

Supplementary). The signal of the protons from the methyl group of hydroxybutyrate ( $\text{CH}_3\text{-HB}$   $\delta$  1.2 ppm) was also used to quantify the number of micromoles of hydroxybutyrate produced by the different enzymes on PHB. This method allowed to compare the amount of PHB and PHBHV11 oligomer released in the supernatant by the enzyme.

The quantity of oligomers released from PHB degradation increased over time for all enzymes, but to a lesser extent for dPhaZ-Apla (Fig. 5A). At the end of the experiment, a total of  $4.63 \pm 0.13$ ,  $14.43 \pm 2.41$  and  $10.44 \pm 1.9$  of HB units were released by dPhaZ-Apla, dPhaZ-Rpic and dPhaZ-Afae, respectively (Fig. 5A). Statistical analysis confirmed difference in oligomer releasing at T40 between the enzymes. Significant differences were found between dPhaZ-Apla vs. dPhaZ-Rpic and dPhaZ-Apla vs. dPhaZ-Afae ( $p < 0.05$ ) demonstrating a better degradation of dPhaZ-Rpic and dPhaZ-Afae compared to dPhaZ-Apla as twice to three time more effective. Although dPhaZ-Rpic seemed to have a slightly better degradation activity than dPhaZ-Afae, no significant difference was highlighted.

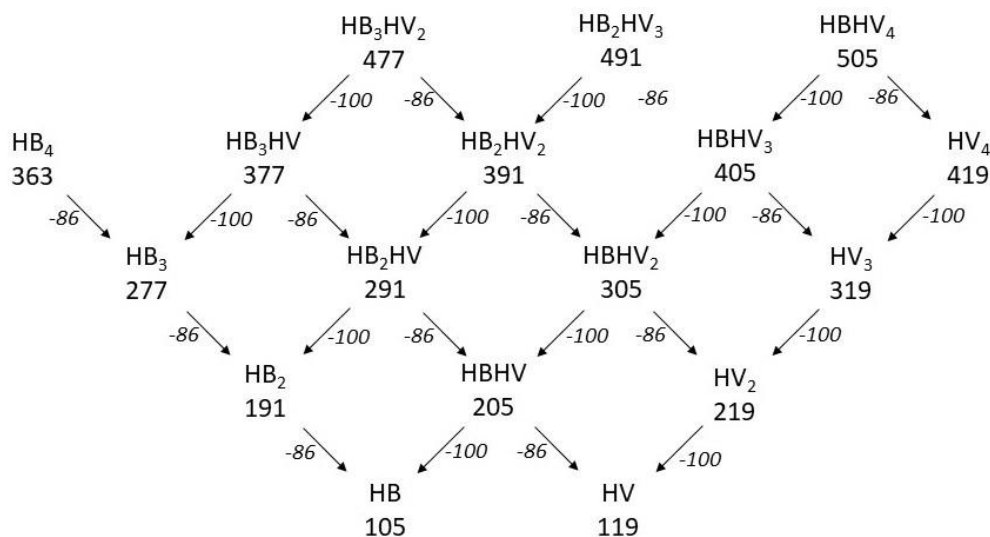


**Figure 5:** Amounts of 3-hydroxybutyrate units released as water soluble products during the enzymatic degradation of PHB (A) and PHBHV11 (B) by dPhaZ-Apla, dPhaZ-Rpic and dPhaZ-Afae.

Similar trends were observed on the degradation of PHBV, with an increase of both HB and HV units over time for all enzymes. It is worth noting that the signal intensity of HB and HV peaks were proportional to the polymer composition (90% of HB ; 10 % of HV) and the production of HV oligomers followed the same kinetics as HB oligomers but with concentrations 10 time lower.

The enzyme dPhaZ-Apla hydrolysed  $9.1 \pm 0.08$   $\mu\text{mol}$  of HB units after 40h and appeared less efficient than dPhaZ-Rpic and dPhaZ-Afae which produced  $25.9 \pm 0.55$  and  $21.2 \pm 0.97$   $\mu\text{mol}$  of HB, respectively. Statistical analysis revealed significant differences between all enzymes showing a better degradation efficiency of dPhaZ-Rpic compared to dPhaZ-Afae and finally to dPhaZ-Apla ( $p < 0.05$ ). Statistical analysis also indicated that PHBHV was significantly more degraded than PHB ( $p < 0.05$ ), as the ratio of HB and HV units was similar to the PHBHV initial proportions and the quantity of released HB units during PHBHV degradation was twice higher.

The composition of PHB and PHBHV11 oligomers was determined by high resolution accurate mass spectrometry (LC-HRMS) (Table 2S and Table 3S, Supplemental data). Mass spectrum interpretation revealed that the degradation of PHB after 40h led to monomers (HB), dimers ( $\text{HB}_2$ ), trimers ( $\text{HB}_3$ ) and tetramers ( $\text{HB}_4$ ) of HB.  $\text{HB}_4$  were observed only for the dPhaZ-Apla enzyme, thus confirming a less efficient degradation compared to the other two enzymes. For PHBHV degradation, several oligomers of HB and HV from monomers to pentamers were detected by LC:MS in both negative and positive mode (fig. 4). Identification was confirmed by the used of analytical standards and the presence of several adducts ( $\text{H}^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{NH}_4^+$ ) for each compound. Again, a greater variety of oligomers was noticed for dPhaZ-Apla and from monomers to pentamers, indicating a less efficient cut and therefore longer oligomer chains.



**Figure 6:** Oligomers pattern released and their associated molecular weight used for LC/HRMS identification



## 4. Discussion

### 4.1. Characterization of a novel dPhaZ<sub>SCL</sub> depolymerase from *Alteromonas plasticoclasticus* MED1

One of the originalities of this work was to look at the potential of a marine bacteria isolated from a natural PHA plastsphere for dPhaZ<sub>SCL</sub> depolymerase activities using a four-step process. First, a mature biofilm growing for two months on PHBHV under natural marine conditions has been preselected by transfer on a new media for another two months with PHBHV as sole carbon source (Cheng et al., 2022). Second, the pre-selected biofilm served as an inoculum to isolate a novel bacteria able to biodegrade PHBHV as sole carbon source, called *Alteromonas plasticoclasticus* MED1 (Barbe et al., submitted, Annexe F). Third, the sequenced genome of this strain revealed the presence of phaZ gene (ALSP\_2657) annotated as an external dPhaZ<sub>SCL</sub> depolymerase. Transcriptomic analysis confirmed the activity of the dPhaZ<sub>SCL</sub> protein that was 30 times more expressed in PHBHV degradation conditions than in glucose culture conditions (Barbe et al. submitted, Annexe F). Isolation of bacteria originated from the plastsphere on the selected polymer has been described as right direction to better fit with natural conditions (Jacquin et al. 2019), but poorly followed by actions. Here, we presented the fourth step by producing and characterizing the dPhaZ<sub>SCL</sub> enzyme from the marine *Alteromonas plasticoclasticus* MED1. Comparison was possible with two other phaZ genes from *Ralstonia pickettii* T1 and *Alcaligenes faecalis* AE122, isolated from an activated sludge (Shirakura et al., 1986) or from coastal seawater (Kita et al., 1997), respectively. We believe that the success of our experimental strategy opens new routes to discover new dPhaZ<sub>SCL</sub> depolymerase from the natural plastsphere.

In this study, comparison of amino acid sequences revealed the presence of a catalytic domain of type I with the conserved catalytic triad consisting of Histidine – Aspartic acid – Histidine, already described in *Ralstonia pickettii* T1 and *Alcaligenes faecalis* AE122 (Jendrossek, 2001; Kita et al., 1997). SBD of dPhaZ-Apla was divided into two types, as previously described in the marine bacterium *Alcaligenes faecalis* AE122 (Kita et al., 1997). Besides, homology degree of the SBD Type II between the two proteins were over 78.2%. Amino acid sequence structure of dPhaZ-Apla showed typical characteristics found in dPhaZ<sub>SCL</sub> and more particularly to the one found in *Alcaligenes faecalis* AE122.

Turbidity tests showed a slightly less activity of dPhaZ-Apla when compared to dPhaZ-Rpic and dPhaZ-Afae produced from *R. pickettii* T1 and *A. faecalis* AE122, respectively. The dPhaZ-Apla showed a very rapid activity during the first 4.5 hours of incubation with PHBHV powder, which

decreased more rapidly than the dPhaZ-Rpic and dPhaZ-Afae activities that remained almost stable until 7 hours. A classical reason for the reaction slowdown could be that the substrate became limiting or that reaction conditions are not optimal. Since the substrate concentration was not limiting for the other two dPhaZ-Rpic and dPhaZ-Afae enzymes in the same conditions, we hypothesize that the second hypothesis is more relevant. Further studies will be needed to evaluate the main factors driving the dPhaZ-Apla activity, such as temperature, buffer composition, pH, enzyme concentration, and the presence of any inhibitors or activators. Preliminary tests were done in our study to evaluate the importance of enzyme concentration, since it has been previously shown as a critical aspect for *scl*-PHA degradation (Mukai et al., 1993a, 1993b). These authors showed that at low concentrations, the enzyme does not recover all the polymer surface and lack of efficiency. At high concentrations, the catalytic domain of the enzyme is not capable of hydrolysing PHA molecules on the surface due to complete covering of the enzymes through the binding site. Our preliminary tests showed an optimal concentration of 1 µg for dPhaZ-Apla, dPhaZ-Rpic and dPhaZ-Afae in our experimental conditions. It has to be noted that precise concentration of dPhaZ-Apla could not be determined because of a second band visible on the gel after the protein purification, thus indicating that the protein was not 100% pure (Fig. 2S). Nevertheless, preliminary tests did not show any difference of kinetics between 1, 5 and 10 µg of enzyme addition on PHBHV powder. Deeper analysis of the degradation of various *scl*-PHA and of the degradation products helped to go deeper on the rationale of lower enzyme activity of dPhaZ-Apla (see below).

#### 4.2. Impact of *scl*-PHA composition and properties on enzymatic degradation

In this study, we also compared the action of the three dPhaZ<sub>SCL</sub> on two *scl*-PHA with different composition and properties. We observed that dPhaZ-Apla depolymerase presented higher degradation kinetics on the PHBHV than on PHB, as shown by both SEM and <sup>1</sup>H-NMR analysis. Such difference might be explained by the repartition of lamellar crystalline and amorphous regions in those polymers. This hypothesis was reinforced by the fact that the same tendency was also found with dPhaZ-Rpic and dPhaZ-Afae depolymerase in the same experimental conditions. Previous studies showed that the dPhaZ-Rpic enzyme firstly hydrolyses the PHB chains in the amorphous state on the surface of the films and subsequently erodes the PHB chains in the crystalline state (Kumagai et al., 1992). The PHBHV structure being more amorphous ( $\Delta H_m = 40 \text{ J.g}^{-1}$ ) than PHB ( $\Delta H_m = 87 \text{ J.g}^{-1}$ ), higher dPhaZ<sub>SCL</sub> activity may be directly linked with the amorphous to crystalline ratio of the polymer type. Our results reinforce the idea that the mobility of the chains in the amorphous state play an important role in *scl*-PHA enzymatic degradation, by giving a comfortable accessibility to the catalytic domain (Kikkawa et al., 2005 ; Numata et al., 2009, 2005).

Special interest given on the degradation products by  $^1\text{H-NMR}$  also provided new insights for the differential degradation of PHB and PHBHV by the the dPhaZ<sub>SCL</sub>. First, whatever the enzyme used for degradation on the polyester films (dPhaZ-Apla, dPhaZ-Rpic and dPhaZ-Afae), the quantity of released HB units were always higher on PHBHV than on PHB. The quantities of HB units released were almost twice more important on PHBHV than on PHB. Interestingly, the ratio of signal intensities of HB and HV units released during PHBHV degradation was proportional to the PHBHV composition ratio (90% HB and 10% HV). This original result indicates a random cut of the polymer chain by all the dPhaZ<sub>SCL</sub> enzymes, with a non-specificity to degrade either the HB or the HV pattern units. Second, we observed that the amount of oligomers released by dPhaZ-Apla depolymerase was lower than for the dPhaZ-Rpic and dPhaZ-Afae enzymes, whatever the polymer type. Analysis of the oligomer compositions revealed that the enzymatic degradation by dPhaZ-Apla produced various oligomer structures, with higher production of pentamers, tetramers and trimers when compared to the more efficient dPhaZ-Afae and dPhaZ-Rpic that produced mainly monomers. Difference in efficiency between enzymes could result from a different hydrophobic nature of the binding site leading to lower adsorption of the enzyme to the polymer surface (Mukai et al., 1993a, 1993b). However, dPhaZ-Apla and dPhaZ-Afae have similar SBD structures. Optimization of the experimental conditions such as pH, temperature, buffers or inhibitors need to be further explored to exploit the full potential of dPhaZ-Apla.

## 5. Conclusion

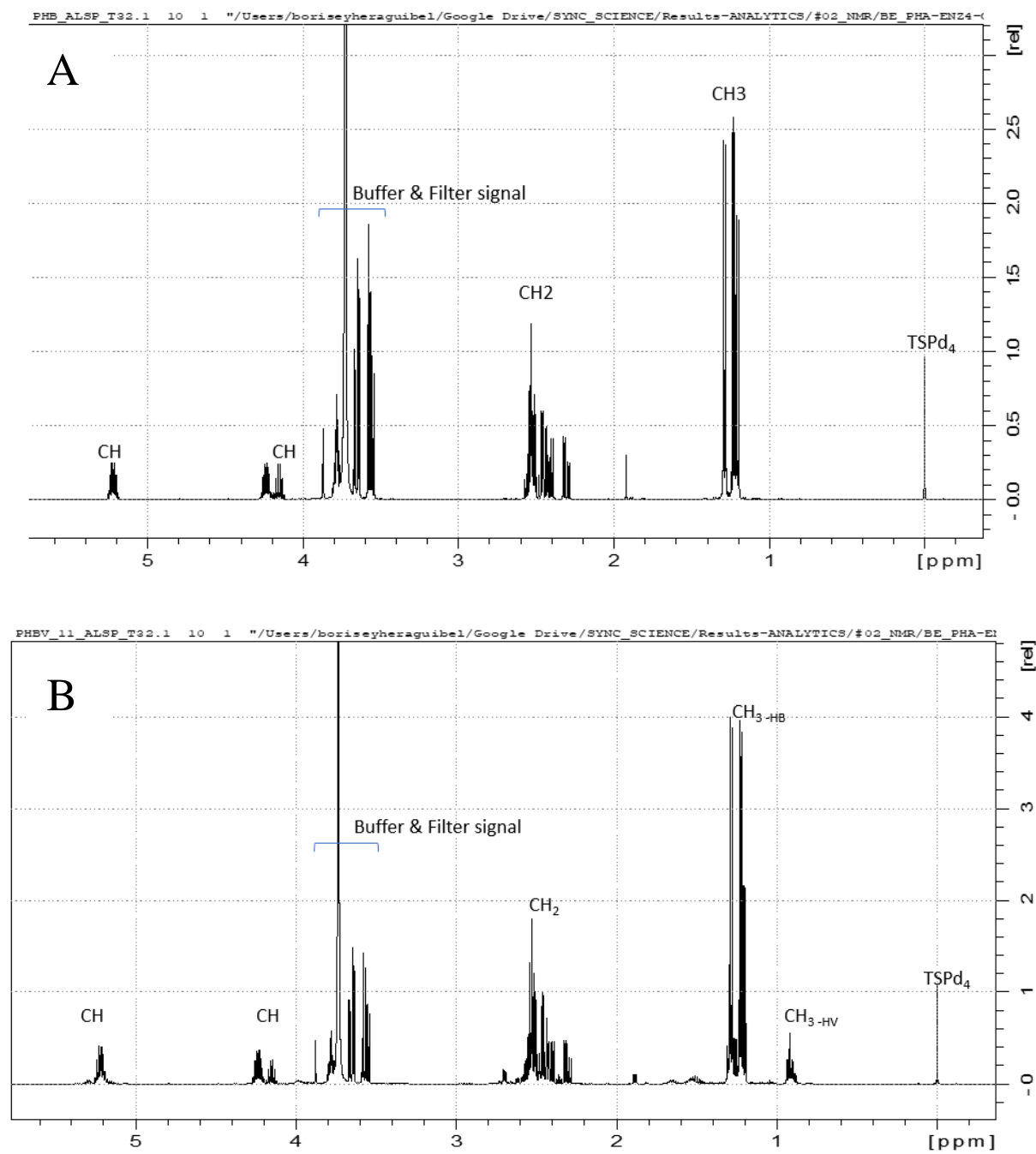
In this study, we presented the production and the characterization of a novel extracellular *scl*-PHA depolymerase dPhaZ-Apla from the marine bacterium *Alteromonas plasticoclasticus* MED1. We believe that the experiment design to isolate this strain from a natural biofilm growing on PHA is a new route for further discovery of new dPhaZ<sub>SCL</sub> enzymes. The dPhaZ-Apla presented a catalytic of type I and two SBD. It also showed lower degradation rates than other dPhaZ<sub>SCL</sub> enzymes, i.e. dPhaZ-Rpic and dPhaZ-Apla enzymes from *Ralstonia pickettii* T1 and *Alcaligenes faecalis* AE122, respectively. This result was reinforced by the detection of various oligomer structures (but not more than pentamers) with dPhaZ-Apla depolymerase, which contrasted with the more efficient dPhaZ-Afae and dPhaZ-Rpic that produced mainly monomers. Further studies are needed to optimize the experimental conditions of dPhaZ-Apla to highlight an optimal enzymatic activity. dPhaZ-Apla depolymerase showed similar specificity than dPhaZ-Afae and dPhaZ-Rpic enzymes, with higher degradation of the more amorphous PHBHV than the more crystalline PHB. The enzymes also showed a random cut of the polymer chain, with no distinction between the HB or the HV pattern units.

## References

- Blevins, H.M., Blue, M.K., Cobbs, B.D., Ricotilli, T.A., Kyler, S.L., Shuey, C.T., Thompson, W.D., Baron, S.F., 2018. Characterization of an Extracellular Polyhydroxyalkanoate Depolymerase from *Streptomyces* sp. SFB5A. *J Bioremediat Biodegrad* 09. <https://doi.org/10.4172/2155-6199.1000452>
- Brandi, H., Bachofen, R., Mayer, J., Wintermantel, E., 1995. Degradation and applications of polyhydroxyalkanoates. *Can. J. Microbiol.* 41, 143–153. <https://doi.org/10.1139/m95-181>
- Cheng, J., Eyheraguibel, B., Jacquin, J., Pujo-Pay, M., Conan, P., Barbe, V., Hoypierres, J., Deligey, G., Halle, A.T., Bruzaud, S., Ghiglione, J.-F., Meistertzheim, A.-L., 2022. Biodegradability under marine conditions of bio-based and petroleum-based polymers as substitutes of conventional microparticles. *Polymer Degradation and Stability* 206, 110159. <https://doi.org/10.1016/j.polymdegradstab.2022.110159>
- Eyheraguibel, B., Traikia, M., Fontanella, S., Sancelme, M., Bonhomme, S., Fromageot, D., Lemaire, J., Lauranson, G., Lacoste, J., Delort, A.M., 2017. Characterization of oxidized oligomers from polyethylene films by mass spectrometry and NMR spectroscopy before and after biodegradation by a *Rhodococcus rhodochrous* strain. *Chemosphere* 184, 366–374. <https://doi.org/10.1016/j.chemosphere.2017.05.137>
- Jendrossek, D., 2001. Microbial degradation of polyesters. *Adv. Biochem. Eng. Biotechnol.* 71, 293–325. [https://doi.org/10.1007/3-540-40021-4\\_10](https://doi.org/10.1007/3-540-40021-4_10)
- Jendrossek, D., Schirmer, A., Schlegel, H.G., 1996. Biodegradation of polyhydroxyalkanoic acids. *Appl Microbiol Biotechnol* 46, 451–463. <https://doi.org/10.1007/s002530050844>
- Kedzierski, M., D’Almeida, M., Magueresse, A., Le Grand, A., Duval, H., César, G., Sire, O., Bruzaud, S., Le Tilly, V., 2018. Threat of plastic ageing in marine environment. Adsorption/desorption of micropollutants. *Marine Pollution Bulletin* 127, 684–694. <https://doi.org/10.1016/j.marpolbul.2017.12.059>
- Khosravi-Darani, K., Mokhtari, Z.-B., Amai, T., Tanaka, K., 2013. Microbial production of poly(hydroxybutyrate) from C1 carbon sources. *Appl Microbiol Biotechnol* 97, 1407–1424. <https://doi.org/10.1007/s00253-012-4649-0>
- Kikkawa, Y., Yamashita, K., Hiraishi, T., Kanesato, M., Doi, Y., 2005. Dynamic Adsorption Behavior of Poly(3-hydroxybutyrate) Depolymerase onto Polyester Surface Investigated by QCM and AFM. *Biomacromolecules* 6, 2084–2090. <https://doi.org/10.1021/bm0500751>
- Kita, K., Mashiba, S., Nagita, M., Ishimaru, K., Okamoto, K., Yanase, H., Kato, N., 1997. Cloning of poly(3-hydroxybutyrate) depolymerase from a marine bacterium, *Alcaligenes faecalis* AE122, and characterization of its gene product. *Biochim Biophys Acta* 1352, 113–122. [https://doi.org/10.1016/s0167-4781\(97\)00011-0](https://doi.org/10.1016/s0167-4781(97)00011-0)
- Knoll, M., Hamm, T.M., Wagner, F., Martinez, V., Pleiss, J., 2009. The PHA Depolymerase Engineering Database: A systematic analysis tool for the diverse family of polyhydroxyalkanoate (PHA) depolymerases. *BMC Bioinformatics* 10, 89. <https://doi.org/10.1186/1471-2105-10-89>
- Koller, M., Salerno, A., Dias, M.M. de S., Reiterer, A., Braunegg, G., 2010. Modern Biotechnological Polymer Synthesis: A Review. *Food technology and biotechnology* 48, 255–269.
- Kumagai, Y., Kanesawa, Y., Doi, Y., 1992. Enzymatic degradation of microbial poly(3-hydroxybutyrate) films. *Makromol. Chem.* 193, 53–57. <https://doi.org/10.1002/macp.1992.021930105>
- Laycock, B., Halley, P., Pratt, S., Werker, A., Lant, P., 2014. The chemomechanical properties of microbial polyhydroxyalkanoates. *Progress in Polymer Science, Topical Issue on Biorelated Polymers* 39, 397–442. <https://doi.org/10.1016/j.progpolymsci.2013.06.008>
- Lemechko, P., Le Fellic, M., Bruzaud, S., 2019. Production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) using agro-industrial effluents with tunable proportion of 3-hydroxyvalerate

- monomer units. *International Journal of Biological Macromolecules* 128, 429–434. <https://doi.org/10.1016/j.ijbiomac.2019.01.170>
- Mukai, K., Yamada, K., Doi, Y., 1993a. Enzymatic degradation of poly(hydroxyalkanoates) by a marine bacterium. *Polymer Degradation and Stability* 41, 85–91. [https://doi.org/10.1016/0141-3910\(93\)90066-R](https://doi.org/10.1016/0141-3910(93)90066-R)
- Mukai, K., Yamada, K., Doi, Y., 1993b. Kinetics and mechanism of heterogeneous hydrolysis of poly[(R)-3-hydroxybutyrate] film by PHA depolymerases. *International Journal of Biological Macromolecules* 15, 361–366. [https://doi.org/10.1016/0141-8130\(93\)90054-P](https://doi.org/10.1016/0141-8130(93)90054-P)
- Numata, K., Abe, H., Iwata, T., 2009. Biodegradability of Poly(hydroxyalkanoate) Materials. *Materials* 2, 1104–1126. <https://doi.org/10.3390/ma2031104>
- Numata, K., Kikkawa, Y., Tsuge, T., Iwata, T., Doi, Y., Abe, H., 2005. Enzymatic Degradation Processes of Poly[(R)-3-hydroxybutyric acid] and Poly[(R)-3-hydroxybutyric acid-co-(R)-3-hydroxyvaleric acid] Single Crystals Revealed by Atomic Force Microscopy: Effects of Molecular Weight and Second-Monomer Composition on Erosion Rates. *Biomacromolecules* 6, 2008–2016. <https://doi.org/10.1021/bm0501151>
- Pachekoski, W.M., Agnelli, J.A.M., Belem, L.P., 2009. Thermal, mechanical and morphological properties of poly (hydroxybutyrate) and polypropylene blends after processing. *Materials Research* 12, 159–164. <https://doi.org/10.1590/S1516-14392009000200008>
- Paul-Pont, I., Ghiglione, J.-F., Gastaldi, E., Ter Halle, A., Huvet, A., Bruzard, S., Lagarde, F., Galgani, F., Duflos, G., George, M., Fabre, P., 2023. Discussion about suitable applications for biodegradable plastics regarding their sources, uses and end of life. *Waste Management* 157, 242–248. <https://doi.org/10.1016/j.wasman.2022.12.022>
- Perchat, N., Saaidi, P.-L., Darii, E., Pellé, C., Petit, J.-L., Besnard-Gonnet, M., De Berardinis, V., Dupont, M., Gimbernat, A., Salanoubat, M., Fischer, C., Perret, A., 2018. Elucidation of the trigonelline degradation pathway reveals previously undescribed enzymes and metabolites. *Proc. Natl. Acad. Sci. U.S.A.* 115. <https://doi.org/10.1073/pnas.1722368115>
- Shirakura, Y., Fukui, T., Saito, T., Okamoto, Y., Narikawa, T., Koide, K., Tomita, K., Takemasa, T., Masamune, S., 1986. Degradation of poly(3-hydroxybutyrate) by poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis* T1. *Biochimica et Biophysica Acta (BBA) - General Subjects* 880, 46–53. [https://doi.org/10.1016/0304-4165\(86\)90118-2](https://doi.org/10.1016/0304-4165(86)90118-2)
- Sudesh, K., Abe, H., Doi, Y., 2000. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Progress in Polymer Science* 25, 1503–1555. [https://doi.org/10.1016/S0079-6700\(00\)00035-6](https://doi.org/10.1016/S0079-6700(00)00035-6)
- Tanio, T., Fukui, T., Shirakura, Y., Saito, T., Tomita, K., Kaiho, T., Masamune, S., 1982. An extracellular poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis*. *Eur J Biochem* 124, 71–77. <https://doi.org/10.1111/j.1432-1033.1982.tb05907.x>
- Wang, Y., Inagawa, Y., Osanai, Y., Kasuya, K., Saito, T., Matsumura, S., Doi, Y., Inoue, Y., 2002. Enzymatic Hydrolysis of Chemosynthesized Atactic Poly(3-hydroxybutyrate) by Poly(3-hydroxyalkanoate) Depolymerase from *Acidovorax* Sp. TP4 and *Ralstonia p ickettii* T1. *Biomacromolecules* 3, 894–898. <https://doi.org/10.1021/bm020052b>
- Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A., 2013. Life in the “Plastisphere”: Microbial Communities on Plastic Marine Debris. *Environ. Sci. Technol.* 47, 7137–7146. <https://doi.org/10.1021/es401288x>

## Supplemental data



**Figure 1S :**  $^1\text{H}$  n.m.r spectra of PHB (A) and PHBHV (B) and peak assignments

**Table 1S:** Averaged amounts and composition of triplicates of water-soluble products during the enzymatic degradation of PHB and PHBV11 films by dPhaZ-Apla, dPhaZ-Rpic and dPhaZ-Afae depolymerases.

PHB				PHBV		
CH3-HB ( $\mu\text{mol}$ )	dPhaZ-Rpic	dPhaZ-Afae	dPhaZ-Apla	dPhaZ-Rpic	dPhaZ-Afae	dPhaZ-Apla
8	3.79	3.74	2.08	5.16	4,08	3,36
16	7.74	6.87	2.39	13.85	5,88	5,28
24	8.47	6.50	4.14	15.48	12,52	7,92
32	9.73	9.60	4.96	21.40	14,17	7,42
40	12.93	10.44	4.63	25.92	21,25	9,09
CH3-HV ( $\mu\text{mol}$ )				dPhaZ-Rpic	dPhaZ-Afae	dPhaZ-Apla
8				0.54	0.42	0.35
16				1.44	0.61	0.55
24				1.67	1.30	0.82
32				2.27	1.51	0.77
40				2.73	2.21	0.95

**Table 2S:** Oligomer compositions at 40 hours for PHB enzymatic degradation

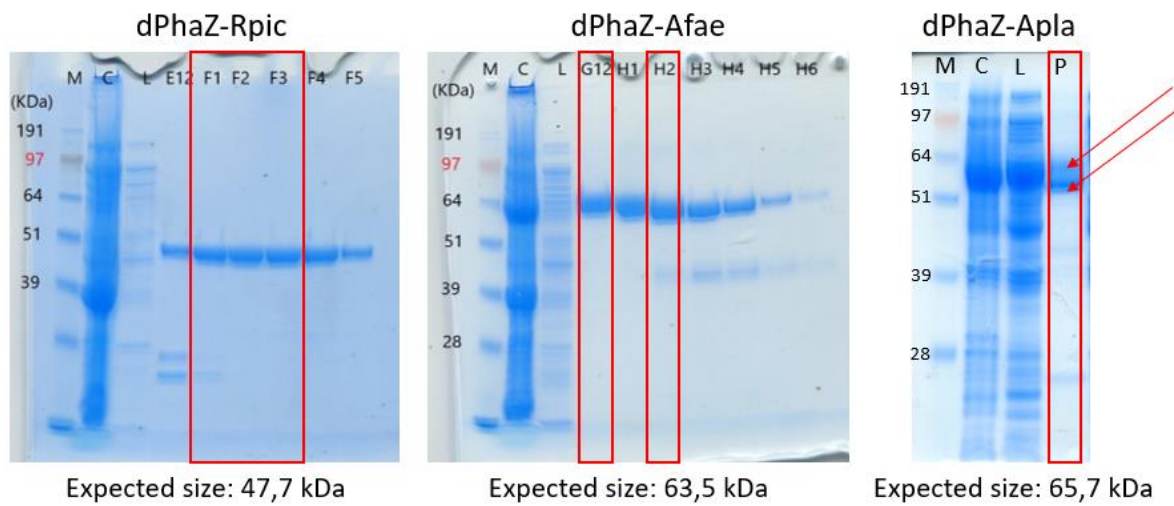
	dPhaZ-Apla	dPhaZ-Rpic	dPhaZ-Afae
HB1	19 692 830	24 000 334	-
HB2	60 536 664	66 008 076	134 803 392

HB3	18 016 894	11 030 649	613 679
HB4	7 190	-	

**Table 3S:** Oligomer compositions at 40 hours for PHBHV enzymatic degradation

PHBHV	dPhaZ-Apla	dPhaZ-Rpic	dPhaZ-Afae
HB1	48 431 952	37 912 132	-
HB2	139 391 904	105 090 936	134 803 392
HB3	37 704 312	32 849 382	613 679
HB4	17 740	-	
HV1	-	1 937 165	17 287 686
HV2	3 300 774	2 538 089	13 854 849
HV3	3 598 175	2 359 315	2 710 216
HV4	209 955	34 460	
HB1HV1	34 068 344	22 165 600	47 437 676
HB1HV2	25 578 198	17 337 644	6 669 076
HB1HV3	2 239 685	360 571	
HB1HV4	20 563		
HB2HV1	64 154 592	43 711 696	3 029 993
HB2HV2	5 206 641	1 637 993	-
HB2HV3	29 191		
HB3HV1	1 312 068	513 050	
HB3HV2	33 629		





**Figure 2S:** Purification and characterization of the dPhaZ: red rectangle indicates purified fractions used in this study. Red arrow indicates the two band found in the purified fraction of dPhaZ-Apla used in this study.

# Chapitre 4 : Une nouvelle bactérie marine capable de synthétiser des polyhydroxyalcanoates à chaînes courtes (scl-PHA) et moyennes (mcl-PHA)

## Préambule

Ce chapitre sous forme d'article scientifique en anglais est une étude qui décrit pour la première fois la production de *scl*- et *mcl*-PHA par une souche bactérienne marine, *Alteromonas plasticoclasticus* MED1. Cette souche, isolée du milieu naturel à partir d'un biofilm colonisant du PHBHV a déjà montré des capacités à dégradation des *scl*-PHA (article Barbe et al. Soumis, Annexe F). De manière originale, nous nous sommes intéressé à ses capacités de production de PHA, qui peuvent être connexe à la dégradation chez certaines espèces bactériennes. L'analyse de son génome a effectivement révélé la présence de gènes attribuables à des PHA synthases. Nous nous sommes alors intéressé à la capacité de la souche de produire non seulement des *scl*-PHA, mais aussi des *mcl*-PHA. *Alteromonas plasticoclasticus* MED1 a donc été mis en culture avec un milieu enrichi en glucose et en acide nonanoïque. De plus, les gènes intervenant dans les voies métaboliques de production des PHA produits par cette souche marine ont été décrits.

Les objectifs de cette étude sont (1) de caractériser les *scl*-PHA et *mcl*-PHA produits par *Alteromonas plasticoclasticus* MED1 et (2) de confirmer l'utilisation de la voie métabolique de la  $\beta$ -oxydation.

Les résultats présentés proviennent d'une étroite collaboration entre 3 laboratoires multidisciplinaires : l'IRDL, le LOMIC et le Genoscope. Les cultures de la souche marine ont été réalisées en milieu Zobell à partir de différentes sources de carbone (glucose ou acide nonanoïque) en erlenmeyer. Les propriétés thermiques des PHA produits ont été évaluées par calorimétrie différentielle à balayage et leur composition par chromatographie à phase gazeuse. L'analyse des gènes impliqués dans la production de PHA par *Alteromonas plasticoclasticus* MED1 a été réalisée à l'aide de la plateforme « Microscope ».

Les résultats confirment les capacités de synthèse de PHA de la bactérie marine. La souche est capable d'accumuler un PHB et un PHN à partir de sucre et d'acide nonanoïque, respectivement. L'analyse des gènes impliqués et des PHA synthases présentes dans le génome suggèrent l'utilisation de la glycolyse et de la  $\beta$ -oxydation pour la production du PHB et du PHN, respectivement. Ce chapitre décrit, pour la première fois la production de *mcl*-PHA par une bactérie marine isolée de la plastisphère, utilisant la voie de la  $\beta$ -oxydation et non la synthèse *de novo* des acides gras.

# A marine bacteria able to synthesize both short- and medium-chain length polyhydroxyalkanoates

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## Highlights

- *Alteromonas plasticoclasticus* *MEDI* synthesizes *scl*- or *mcl*-PHA depending on the carbon source available.
- *A. plasticoclasticus* *MEDI* genome possess a Class IV PHA synthase for *scl*-PHA production.
- *A. plasticoclasticus* *MEDI* use the  $\beta$ -oxidation pathway through the enoyl-CoA hydratase to produce *mcl*-PHA.

**Keywords:** Polyhydroxyalkanoates, PHA depolymerase, *Alteromonas*

## Abstract

Bioplastics have drawn more interest from researchers as worries about future oil shortages and plastic pollution have grown. The polyhydroxyalkanoates (PHA) family of polyester is a promising group of biosourced and biodegradable bioplastics. No marine bacteria were tested for *scl*- and *mcl*-PHA production together, in particular for *mcl*-PHA production through the  $\beta$ -oxidation pathway. Here, we tested the PHA production of both type of PHA by the marine strain *Alteromonas plasticoclasticus* *MEDI*. Our approach using genomic analysis and culture in erlenmeyer from different carbon sources conducted to the production of PHB from glucose and a PHN from nonanoic

acid. We report here and discuss the discover of the first marine bacterium able to synthetize both *scl*- and *mcl*-PHA with focus on the  $\beta$ -oxidation for *mcl*-PHA production.

## 1 Introduction

Biotechnological potential of marine environment has been increasingly explored in the recent years to identify novel molecules of interest (Imhoff et al., 2011). Among them, the polyhydroxyalkanoates (PHA), which are bio-sourced and biodegradable polymers are produced by many bacteria from various carbon substrates, usually under excess amount of carbon and limited nutrients conditions (Stubbe and Tian, 2003). The PHA constitute a large family and display a wide range of chemical compositions and properties according to the producing strain, the source of carbon used for feeding and the fermentation process. Depending on the carbon source, the bacterial strain and the PHA production process, more than 150 different monomers can compose the PHA family (Steinbüchel and Valentin, 1995). They can be divided into two subgroups: *Short Chain-Length* PHA (*scl*-PHA) composed of 3 to 5 carbon atoms, *Medium Chain-Length* PHA (*mcl*-PHA) composed of 6 to 14 carbon atoms. Due to their chemical structures and morphologies, physical properties drastically differed between *scl*-PHA that are rigid and brittle polymer while *mcl*-PHA are usually more rubbery and elastic (Pérez-Rivero and Hernandez-Raquet, 2017). The key enzyme involved in PHA synthesis is the PHA synthase PhaC (Zher Neoh et al., 2022). This final enzyme involved in the whole PHA synthesis process allowed the polymerization of monomers obtained from substrate accumulation into PHA polymers. The type of monomers incorporated into the radical chain of PHA is determined by the substrate specificity of the PhaC enzyme (Zher Neoh et al., 2022). Some PhaC only incorporate *scl*-PHA monomers while other will only incorporate *mcl*-PHA monomers depending on their class. A few PhaC have been identified to be able to incorporate *scl*- and *mcl*-PHA monomers, but the phenomenon remains unusual (Bhubalan, K. et al., 2010; Foong et al., 2018). Additionnaly to PhaC, other enzymes such as PhaA and PhaB are involved in the *scl*-PHA production, while PhaJ is needed for *mcl*-PHA synthesis through the  $\beta$ -oxidation pathway (Costa et al., 2018; Korotkova and Lidstrom, 2001; Tsuge et al., 2015). *Mcl*-PHA can also be produced through another pathway. Some bacteria are able to transform carbohydrates into fatty acid through the *De novo* synthesis, where the enzyme PhaG allow the polymerisation of monomers by PhaC (Prieto et al., 2016).

Bacteria isolated from soil or activated sludge such as *Cupriavidus necator* and *Pseudomonas putida* (Prieto et al., 2016; Zhila et al., 2021) remain the most studied and are considered as model organisms for the PHA production. In the marine environment, some strains belonging to *Halomonas*, *Vibrio*, *Cobetia* or *Bacillus* genus have been demonstrated to produce *scl*-PHA (Blandón et al., 2020; Hong et al., 2019; Thomas et al., 2019). To date, no marine bacteria have

ever been reported as *mcl*-PHA producer through the  $\beta$ -oxidation pathway. The use of marine bacteria for industrial biotechnology allow interesting features (Yin et al., 2015). Due to their halophilic character, marine bacteria can grow on high salt concentrations and a larger temperature range, thus simplifying the culture conditions and limiting the risk of contamination that lower the process costs (Yin et al., 2015).

In this study, we evaluated the potential of *Alteromonas plasticoclasticus* *MEDI* to synthesize various PHA depending on the carbon substrate for biomass production. We tested the ability of *A. plasticoclasticus* *MEDI* to produce *scl*-PHA but also *mcl*-PHA, depending on the carbon source available. We used batch cultures with different carbon sources for the production of various PHA, which were characterized after synthesis by using Gas Chromatography (GC) and Differential Scanning Calorimetry (DSC). We also used genomic data to describe the potential differences in the genes and metabolic pathways used by *A. plasticoclasticus* *MEDI* in the production of *scl*-PHA and *mcl*-PHA.

## 2 Material and methods

### 2.1. Culture conditions for PHA production by *A. plasticoclasticus* *MEDI*

*A. plasticoclasticus* *MEDI* was kindly provided by LOMIC laboratory that recently isolated this strain from a biofilm growing on PHA in natural marine condition, as previously described (Barbe et al., submitted, Annexe F). Tests on different substrate concentrations for *A. plasticoclasticus* growth revealed that a Zobell media composed of 5 g.L<sup>-1</sup> of Bacto Tryptone (BACTO™, Gibco), 1 g.L<sup>-1</sup> of Yeast extract (Biokar diagnostic), and 20 g/L<sup>-1</sup> of sea salt (Instant Ocean, Aquarium systems), pH 7.5 was the optimal. A preculture of 200 ml in a 500 ml Erlenmeyer flask in Zobell media was realised (8h, 30 °C, 160 rpm) before any experiment from a cryotube previously stored at -80 °C of the strain. All PHA cultures were inoculated with 10% (V / V) of the preculture. *Scl*-PHA cultures were performed in 500 mL Erlenmeyer flasks with a working volume of 200 ml for 33 h, 53 h or 60 h. Glucose at a concentration of 10 or 20 g.L<sup>-1</sup> was added to the medium at 0 h as PHA accumulation substrate. *Mcl*-PHA accumulation were performed in 1 L Erlenmeyer flasks with a working volume of 400 mL for 48 h with the addition of nonanoic acid at a concentration of 1.5 g.L<sup>-1</sup> at 24 h in a batch process or by pulses of 0.45 g.L<sup>-1</sup> at 23 h, 24.5 h, 26 h, 27.5 h and 29 h in a fed-batch process. Temperature was of 30 °C and agitation of 160 rpm for all experiments. Cells were collected at the end of the experiments by centrifugation (12 000 rpm, 12 min, 4 °C). Pellets were frozen and dried in a freeze dryer overnight until constant weight.

## 2.2. Characterization of PHA

Compositions of the produced PHA were determined by gas chromatography (GC). *Scl*-PHA and *mcl*-PHA underwent a propanolysis (Riis and Mai, 1988) and a methanolysis (Furrer et al., 2007), respectively. Briefly, 10 mg of PHA were dissolved in 1 mL of chloroform or dichloromethane and further propyl esterified (*scl*-PHA) with 1 mL of a solution of 1-propanol/37% HCl (8/2, V/V) or methyl esterified (*mcl*-PHA) with 1 mL of MeOH/BF<sub>3</sub> (10% BF<sub>3</sub>, V/V) at 80°C for 20h. After cooling down, distilled water (V/V) was added and the solutions were vortexed. The organic phase was retrieved, dried on MgSO<sub>4</sub>, filtered on glass cotton and samples were injected on a Perkin Elmer Clarus 480 gas chromatograph equipped with a 30 m x 0.32 mm DB-5 column (HP) with splitless injector and flame ionization detector (FID). Oven temperature, ramp and nitrogen flow were measured according to Riis and Mai (1988) and Furrer et al. (2007).

Thermal properties were determined by differential scanning calorimetry (DSC) using a Mettler-Toledo DSC-882 equipment. About 6 mg of PHA were taken from three weeks old casted-films of PHA. Particular attention was given so that all the PHA samples get the same thermal history in order to avoid the induction of different structures, as reported elsewhere (Crétois et al., 2016; Laycock et al., 2014; Xie et al., 2008). PHA samples were equilibrated to 25°C then heated to 190 °C for *scl*-PHA or 80°C for *mcl*-PHA at 10 °C.min<sup>-1</sup> and kept isothermal for 2 min followed by a cooling down to -40 °C for *scl*-PHA or -70°C for *mcl*-PHA at 10 °C.min<sup>-1</sup>. A second isotherm at -40°C or -70°C was kept for 2 min then the sample was heated from -40 to 190 °C or -70°C to 80°C at 10°C.min<sup>-1</sup> according to the PHA type. Melting temperatures and melting enthalpies were measured from the first heating ramp. Glass transition temperatures were measured on the second heating ramp for all PHA and values correspond to the inflection point.

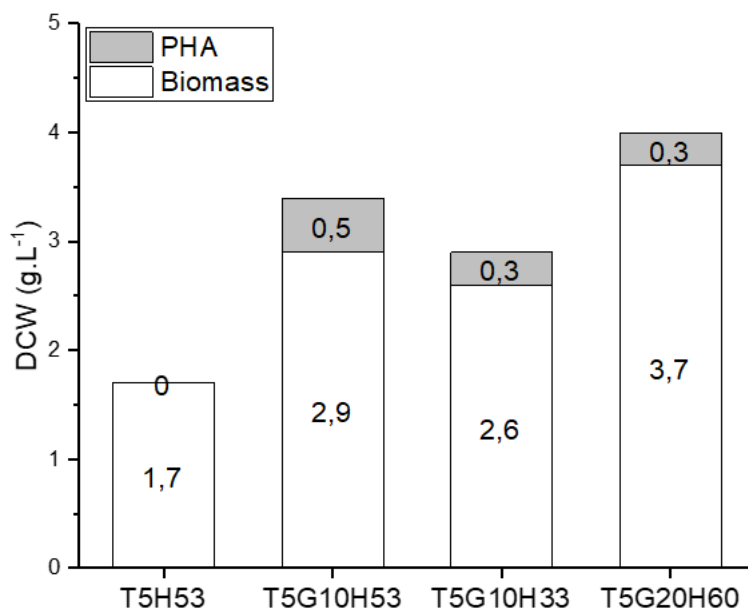
## 2.3. Genomic analysis

The new genus *A. plasticoclasticus* MED1 was isolated from a PHA-plastisphere incubated in Banyuls-sur-mer bay. The complete genome of this strain has been sequenced and transcriptional analysis has highlighted metabolic pathway involved in the biodegradation (Barbe et al, submitted, Annexe F). In parallel with the analysis on biodegradation, a particular focus on the synthesis of PHA was carried out using the tools available in the microbial annotation platform « Microscope » (<https://mage.genoscope.cns.fr/microscope/home/index.php>).

## 3 Results

### 3.1. Production of *scl*-PHA

First, a culture of *A. plasticoclasticus* MED1 without glucose was conducted to a Dry Cell Weight (DCW) of 1.7 g.L<sup>-1</sup> and no PHA was detected after 53 h of culture at 30 °C and 160 rpm (Fig. 1, T5H53). Addition of 10 g.L<sup>-1</sup> at T0 improved the biomass (2.9 g.L<sup>-1</sup>) and triggered the PHA accumulation (0.5 g.L<sup>-1</sup>) for a DCW of 3.4 g.L<sup>-1</sup> at 53 h (Fig. 1, T5G10H53). Same glucose concentration addition at T0 but 33 h of cultures instead of 53 h resulted in a slight lower biomass (2.6 g.L<sup>-1</sup>) and PHA quantity (0.3 g.L<sup>-1</sup>) for a DCW of 2.9 g.L<sup>-1</sup> (Fig. 1, T5G10H33). Double glucose concentration was tested for 60 h (T5G20H60) to improve PHA accumulation. This last culture led to a higher biomass (3.7 g.L<sup>-1</sup>) and a low PHA quantity (0.3 g.L<sup>-1</sup>) for a DCW of 4.0 g.L<sup>-1</sup>. These results suggest that time and substrate concentration do not significantly influence the quantity of accumulated PHA. However, the biomass has been improved by both time and double glucose concentration.



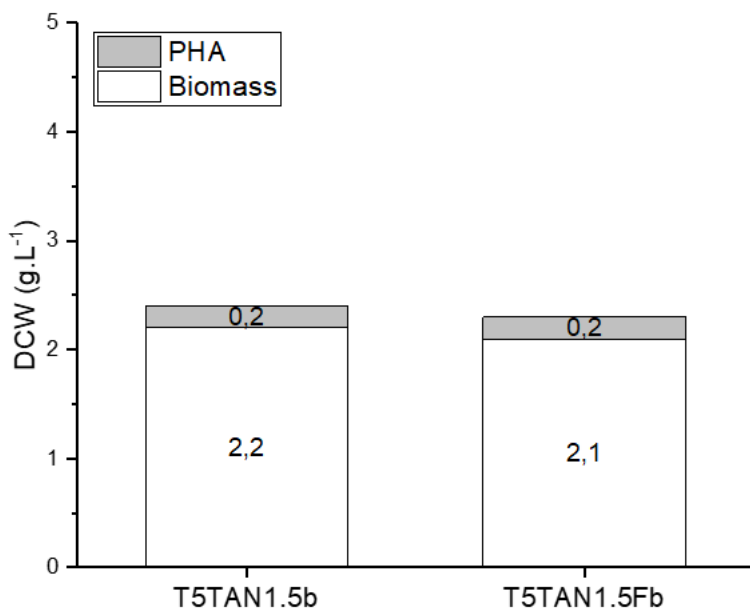
**Figure 1:** Production of *scl*-PHA by *A. plasticoclasticus* MED1 (T5H53 : Tryptone 5 g.L<sup>-1</sup>, 53 h of culture, T5G10H53: Tryptone 5 g.L<sup>-1</sup>, glucose 10 g.L<sup>-1</sup>, 53 h of culture, T5G10H33: Tryptone 5 g.L<sup>-1</sup>, glucose 10 g.L<sup>-1</sup>, 33 h of culture, T5G20H60: Tryptone 5 g.L<sup>-1</sup>, glucose 20 g.L<sup>-1</sup>, 60 h of culture)

### 3.2. Production of *mcl*-PHA

Two feeding strategies have been tested in order to determine the impact of substrate toxicity accumulation. After 24 h of growth in zobell media, 1.5 g.L<sup>-1</sup> of nonanoic acid was added for T5TAN1.5b (Fig. 2). This culture led to a DCW of 2.4 g.L<sup>-1</sup> with a biomass of 2.2 g.L<sup>-1</sup> and a PHA quantity of 0.2 g.L<sup>-1</sup>. The other feeding strategy consisted to add the same nonanoic acid concentration by pulses of 0.45 g.L<sup>-1</sup> of nonanoic acid after 24 h of growth at T23, T245, T26 , T27.5 and T29 in



order to avoid substrate accumulation that can be toxic (T5TAN1.5Fb). A DCW of 2.30 g.L<sup>-1</sup> was reached for a biomass of 2.1 g.L<sup>-1</sup> and a PHA quantity of 0.2 g.L<sup>-1</sup>.



**Figure 2:** Production of *mcl*-PHA by *A. plasticoclasticus* *MEDI* (T5TAN1.5b : Tryptone 5 g.L<sup>-1</sup>, nonanoic acid 1.5 g.L<sup>-1</sup> at T0, 48 h of culture and T5TAN1.5Fb 3: Tryptone 5 g.L<sup>-1</sup>, nonanoic acid 1.5 g.L<sup>-1</sup> by pulses of 100 μL of nonanoic acid (fedbatch), 48 h of culture

### 3.3. Characteristics of PHA produced

Chemical composition and thermal properties of the produced PHA by *A. plasticoclasticus* *MEDI* were analysed by GC and DSC. All *scl*-PHA (T5G10H53, T5G10H33 and T5G20H60) showed the same composition (Table 2). Production from glucose led to the homopolymer PHB. Thermal properties were very similar with a glass transition of ranging from -2 to 4.8°C, a melting temperature of approximately ~179 °C for the three PHB and a melting enthalpy ranging from 74.2 to 91.2 J.g<sup>-1</sup>.

Composition of *mcl*-PHA revealed a 75 and 76 % of HN unit and 25 and 24% of HHp unit contents for T5TAN1.5b and T5TAN1.5Fb, respectively (Table 2). Glass transition, melting temperature and melting enthalpy were very similar for both *mcl*-PHA (- 39.2 and -38.7 °C, 54.8 and 52.7 °C and 17 and 10.7 J.g<sup>-1</sup> for T5TAN1.5b and T5TAN1.5Fb, respectively).

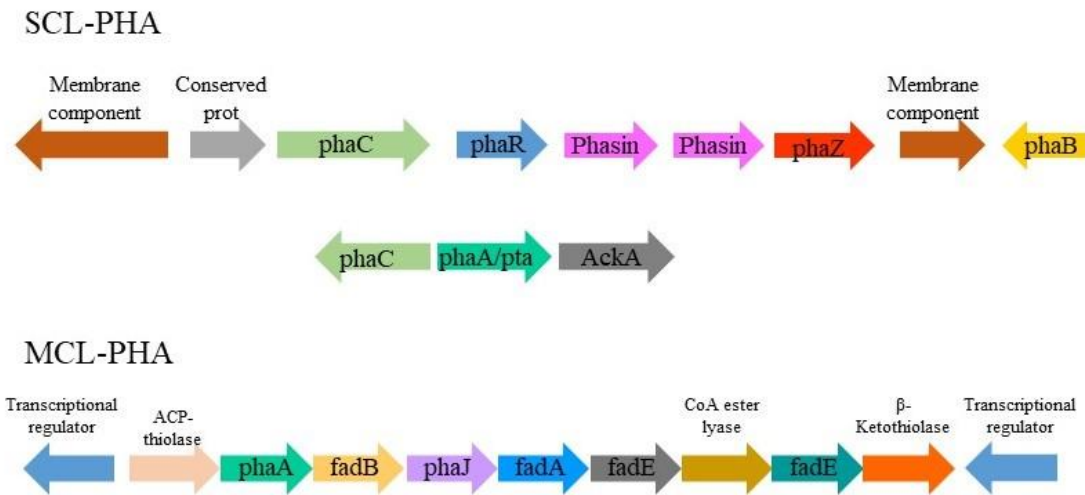
**Table 2:** Composition, average molecular weight and thermal properties of the PHA produced from *A. plasticoclasticus* MED1

	T5G10H53	T5G10H33	T5G20H60	T5TAN1.5b	T5TAN1.5Fb
<b>Glass transition (°C)</b>	-2	4.8	4.5	-39.2	-38,7
<b>Melting temperature (°C)</b>	177.8	179.5	179.5	54.8	52.7
<b>Melting enthalpy ( J.g<sup>-1</sup>)</b>	74.2	91.2	87.9	17	10.7
<b>Composition</b>	HB 100%	HB 100%	HB 100%	HHP 25% HN 75%	HHP 24% HN 76%

### 3.4. Description of the genes involved in *scl*- and *mcl*-PHA degradation

Analysis of the genes involved in the synthesis of *scl*- and *mcl*- of PHA revealed two distinct main groups of genes (Fig. 3). The main gene cluster involved in the *scl*-PHA production brought together nine genes coding for membrane components, phasins, internal depolymerase, and the couple acetoacetyl-CoA reductase (PhaB) / PHA synthase (PhaC) directly involved in the polymer formation. Two copies of acetyl-CoA acetyltransferase (PhaA) were found in genome, allowing the condensation of two acetyl-CoA that correspond to the initial step of the *scl*-PHA synthesis, but not in the operon (Fig. 3).

Another cluster constituted by nine genes surrounded by two transcriptional regulators corresponded to the *mcl*-PHA production (Fig. 3). These genes organized in operon are coding for  $\beta$ -oxidation proteins. The *PhaJ* gene leading to the (R)-3-hydroxyacyl-CoA, precursor of *mcl*-PHA was located in this operon. No *PhaG* gene, coding for a transacylase was identified in the genome of *A. plasticoclasticus* MED1.



**Figure 3:** Genes of *A. plasticoclasticus* MED1 involved in *scl*- and *mcl*-PHA and their relative positions

## 4 Discussion

### 4.1. A marine strain able to produce *scl*- and *mcl*-PHA

One originality of this work was to test the ability of PHA production by *A. plasticoclasticus* MED1, which have been initially described as a PHA-degrader (Barbe et al., submitted, Annexe F). This strain was isolated from the plastisphere that constitutes a unique reservoir of strains with polymer biodegradation abilities that are very different from the surrounding waters (Odobel et al., 2021; Zettler et al., 2013). Studies on production of PHA and extracellular biodegradation of PHA are rarely analysed within a same species at the exception of *Bacillus* sp. (Ma et al., 2011; Ray and Kalia, 2017). *A. plasticoclasticus* MED1 degradation and production capacities deserve to be noticed and further studied. Although concomitant capacities to produce and degrade PHA are not always associated, strains originated from the platisphere identified as potential PHA degraders could represent a new niche for PHA production bacteria.

To our knowledge, genome and PHA production capacities in *Alteromonas* genus have been poorly studied. Only one recent study reported the *phaC* gene presence in *Alteromonas pelagimontana* 5.12<sup>T</sup> but concrete production has not been tested (Sinha et al., 2021). Although marine strains such as *Halomonas* sp., *Vibrio* sp., *Cobetia* sp. or *Bacillus* sp. demonstrated *scl*-PHA production abilities (Blandón et al., 2020; Hong et al., 2019; Thomas et al., 2019), none of them were tested for *mcl*-PHA production from fatty acid. This last ability has only been shown for years by the undefeatable bacteria belonging to *Pseudomonas* genus (Escapa et al., 2011; Sun et al., 2007). We here report the use of genomic tools to predict PHA synthesis abilities as a new method for rapid screening of *mcl*-PHA production abilities in already sequenced bacteria.

#### 4.2. Production efficiency of *scl*- and *mcl*-PHA in batch conditions by *A. plasticoclasticus*

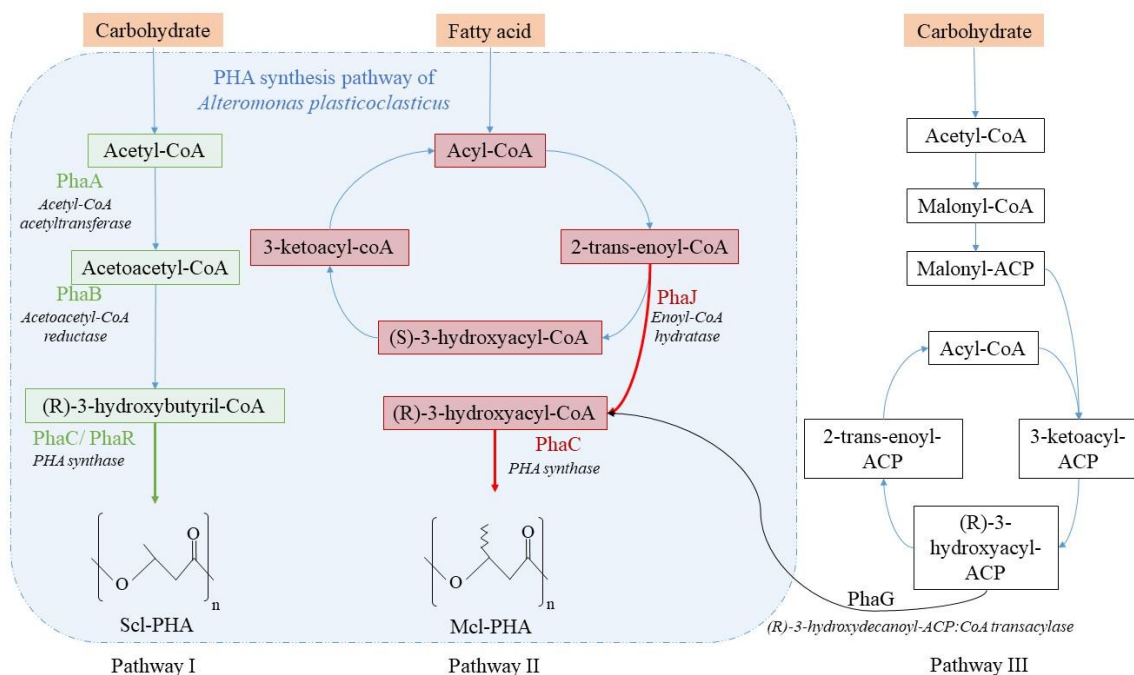
Analysis of physico-chemical properties of the PHA produced confirm the nature of the PHA belonging to a PHB when glucose is used as a carbon source and belonging to PHN when nonanoic acid is used as a carbon source. Chemical composition and thermal properties of those two PHA are common to PHA produced from glucose by *Halomonas* sp. SF2003 (Lemechko et al., 2019) and from nonanoic acid by *Pseudomonas putida* KT2440 (Sun et al., 2007). Nonetheless, we are aware that production yields are willing to be improved when compared to other studied bacteria using the same biosynthesis pathways (Lemechko et al., 2019; Sun et al., 2007). In laboratory conditions, cultures of 24 h of *Halomonas* sp. SF2003 in the same conditions (media, temperature, carbon sources) reached a DCW of 2.4 g.L<sup>-1</sup>, with a PHA content of 34 % (data not shown). *A. plasticoclasticus* MED1 only reached a PHA content of 14% in 48 h. Likewise, *mcl*-PHA production from nonanoic acid with *Pseudomonas putida* KT2440 in Erlenmeyer flask showed a PHA content of 18 % (data not shown). *A. plasticoclasticus* MED1 accumulated only 10 % of PHA for a same DCW. Process parameters to improve the production yield such as stress to trigger accumulation, other growth and accumulation substrates were not explored in this study but constitute the main perspectives to improve PHA content in *A. plasticoclasticus* MED1 cultures.

#### 4.3. Putative metabolic pathways involved in *scl*- and *mcl*-PHA by *A. plasticoclasticus*

Both genomic analysis and PHA production allowed a precise description of metabolic pathway involved in *A. plasticoclasticus* MED1 PHA production. Indeed, two operons with PHA synthesis genes were highlighted in the strain genome. Genes related to *scl*-PHA production such as *phaC* and *phaR* constitute a PHA synthase of class IV (Rehm, 2003). This type of PHA synthase was first described in *Bacillus megaterium* for the production of *scl*-PHA (McCool and Cannon, 2001; Satoh et al., 2002). Compared to PHA synthases of class I or II, PHA synthases of class IV are smaller (40 kDa vs. ~60 kDa for class I) and does not have a hydrophobic C-terminus. PhaR (22 kDa) association is therefore required to PhaC for PHA synthase activity by mediating contact with the hydrophobic polyester core (Rehm, 2003). Additionally, analysis of physico-chemical properties of the PHA produced from glucose by *A. plasticoclasticus* MED1 confirm the nature of the PHA corresponding to a PHB. As described in figure 4, the glucose implemented during *A. plasticoclasticus* MED1 cultures was metabolized through the pathway I. From the glycolyse, the glucose is transformed in

acetyl-CoA that PhaA and PhaB can transform into (R)-3-hydroxybutyryl-CoA. Final polymerization into *scl*-PHA is realized by the two subunits of the PHA synthase PhaC/PhaR.

Genes related to *mcl*-PHA production such as *phaJ* identified in *A. plasticoclasticus MED1* were located in an operon with other genes related to the  $\beta$ -oxidation. Additionally, a second gene coding for a PhaC was identified. PHA synthesis of *mcl*-PHA is realized by PHA synthase of class II (Rehm, 2003). The production of *mcl*-PHA through the  $\beta$ -oxidation pathway (pathway II, Fig. 4) is the most effective way to produce *mcl*-PHA and is realized from fatty acids also called “related” substrates meaning that the monomeric composition of the *mcl*-PHA will be mainly composed of the substrate. This was the case with *A. plasticoclasticus MED1 mcl*-PHA cultures. Production from nonanoic acid led to a *mcl*-PHA mainly composed of HN units but also of HHp because  $\beta$ -oxidation cycle involves a subtraction of even number of carbon atoms substrate (Anjum et al., 2016). This pathway was well studied in *Pseudomonas putida* KT2440 (Prieto et al., 2016). The pathway III (Fig. 4) is another pathway conducting to *mcl*-PHA synthesis. From carbohydrates, due to the *de novo* fatty acid synthesis, the sugar is transformed into (R)-3-hydroxyacyl-ACP and PhaG, a (R)-3-hydroxydecanoyl-ACP: CoA transacylase transforms it into (R)-3-hydroxyacyl-CoA molecule that the PHA synthase PhaC of class II is able to polymerize. This *mcl*-PHA synthesis pathway is “unrelated” to the substrate and conducts to *mcl*-PHA mainly composed of HO and HD units (Davis et al., 2013). The gene coding for PhaG is well identified in *Pseudomonas putida* KT2440 (Davis et al., 2013; Prieto et al., 2016) but not in *A. plasticoclasticus MED1* confirming the use of the fatty acid for *mcl*-PHA synthesis through the  $\beta$ -oxidation metabolic pathway and the glucose for *scl*-PHA synthesis through the glycolysis pathway.



**Figure 4:** Metabolic pathways of *scl*- and *mcl*-PHA synthesis. Blue rectangle highlight pathway used by *A. plasticoclasticus* and the enzymes involved.

Some studies already showed the potential of *scl*-PHA production marine bacteria such as strains belonging to *Halomonas*, *Vibrio*, *Cobetia* or *Bacillus* genus (Blandón et al., 2020; Hong et al., 2019; Thomas et al., 2019). However, studies on *mcl*-PHA production from marine bacteria remain limited. A bacterial strain isolated in the marine environment of French Polynesia, belonging to *Enterobacter* genus was described as a *mcl*-PHA producer (Wecker et al., 2015). However, the substrate used for the production and the final composition revealed that this strain produced *mcl*-PHA from the pathway III (Fig. 4) and not through the  $\beta$ -oxidation pathway. Although, this *de novo* synthesis pathway display some advantage for *mcl*-PHA production by using inexpensive carbon substrate such as glucose, fructose or sucrose (Anjum et al., 2016), it also limits the yield by adding an additional step to transform the sugar into fatty acid.

We here describe the first marine bacteria producing *mcl*-PHA from fatty acid through the  $\beta$ -oxidation pathway. Furthermore, the specific organization of the gene involved in PHA synthesis into operon makes of *A. plasticoclasticus* *MEDI* a luxury model marine organism for further engineering of organism to produce new type of PHA (Escapa et al., 2011).

## 5 Conclusion

Strains producing PHA are usually divided into two groups according to the type of produced PHA; either *scl*- or *mcl*-PHA. In this study we report the production of a *scl*-PHA, i.e. PHB and a *mcl*-PHA, i.e. PHN mainly composed of HN monomer content by a novel marine strain belonging to *A. plasticoclasticus* *MED1*. Genomic analysis together with this production revealed a straight assimilation of sugars and fatty acid through the glycolysis and  $\beta$ -oxidation metabolic pathways, respectively. *A. plasticoclasticus* *MED1* possesses two operons each involved in *scl*- and *mcl*-PHA that could easily be cloned for optimizing production. This strain is the first strain to be described as accumulating both type of PHA and the first marine strain to produce *mcl*-PHA through the  $\beta$ -oxidation pathway from fatty acid. Properties associated to the PHA produced in this study are clearly in line with similar PHA produced by *scl*-PHA producing strain *Halomonas* sp. *SF2003* and by *mcl*-PHA producing strain *Pseudomonas putida* *KT2440*. However, more appropriate growth or accumulating substrate can be used to enhance PHA content in *A. plasticoclasticus* *MED1* culture. Triggering a stress such as nitrogen or phosphorus limitation is also an effective method to improve the quantity of PHA in the cultures (Ahn et al., 2015; Korkakaki et al., 2017). Although these process parameters, as other growth and accumulation substrates were not explored in this study, they constitute the main perspectives to improve PHA contents in *A. plasticoclasticus* *MED1* cultures. Furthermore, these concomitant results on PHA production by *A. plasticoclasticus* *MED1* suggest that the plastisphere could be a new reservoir for isolation of bacteria with PHA-accumulating capacities. Additionally, genomic tools can be used as a method for rapid screening of genes involved in *scl*- and/or *mcl*-PHA production.

However, this first production of both *scl*- and *mcl*-PHA by *A. plasticoclasticus* *MED1* opens new routes to produce new copolymers of PHA, never obtained until now, from co-substrates through straight and efficient metabolic pathways. This novel strain could make it possible to design PHA architectures which are currently inaccessible by conventional fermentation approaches using traditional bacteria. PHA structures combining *scl*-PHA sequences and other *mcl*-PHA sequences could be imagined, which would open the promising way to PHA with characteristics and properties unknown until now.

## References

- Ahn, J., Jho, E.H., Nam, K., Ahn, J., Jho, E.H., Nam, K., 2015. Effect of C/N ratio on polyhydroxyalkanoates (PHA) accumulation by *Cupriavidus necator* and its implication on the use of rice straw hydrolysates. *Environmental Engineering Research* 20, 246–253. <https://doi.org/10.4491/eer.2015.055>
- Anjum, A., Zuber, M., Zia, K.M., Noreen, A., Anjum, M.N., Tabasum, S., 2016. Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: A review of recent advancements. *International Journal of Biological Macromolecules* 89, 161–174. <https://doi.org/10.1016/j.ijbiomac.2016.04.069>
- Bhubalan, K., Kam, Y. C., Yong, K. H., Sudesh, K., 2010. Cloning and expression of the PHA synthase gene from a locally isolated *Chromobacterium* sp. USM2. *MJM*. <https://doi.org/10.21161/mjm.21809>
- Blandón, L., Alvarado-Campo, K.L., Patiño, A.D., Jiménez-Vergara, E., Quintero, M., Montoya-Giraldo, M., Jutinico-Shubach, L.M., Santos-Acevedo, M., Gómez-León, J., 2020. Polyhydroxyalkanoate Production from Two Species of Marine Bacteria: A Comparative Study. *J Polym Environ* 28, 2324–2334. <https://doi.org/10.1007/s10924-020-01770-3>
- Costa, J.A.V., Moreira, J.B., Lucas, B.F., Braga, V.D.S., Cassuriaga, A.P.A., Morais, M.G.D., 2018. Recent Advances and Future Perspectives of PHB Production by Cyanobacteria. *Industrial Biotechnology* 14, 249–256. <https://doi.org/10.1089/ind.2018.0017>
- Crétois, R., Chenal, J.-M., Sheibat-Othman, N., Monnier, A., Martin, C., Astruz, O., Kurusu, R., Demarquette, N.R., 2016. Physical explanations about the improvement of PolyHydroxyButyrate ductility: Hidden effect of plasticizer on physical ageing. *Polymer, Polymers at Interfaces: Probing Mechanics and Interactions by Atomic Force Microscopy* 102, 176–182. <https://doi.org/10.1016/j.polymer.2016.09.017>
- Davis, R., Kataria, R., Cerrone, F., Woods, T., Kenny, S., O'Donovan, A., Guzik, M., Shaikh, H., Duane, G., Gupta, V.K., Tuohy, M.G., Padamatti, R.B., Casey, E., O'Connor, K.E., 2013. Conversion of grass biomass into fermentable sugars and its utilization for medium chain length polyhydroxyalkanoate (*mcl*-PHA) production by *Pseudomonas* strains. *Bioresource Technology* 150, 202–209. <https://doi.org/10.1016/j.biortech.2013.10.001>
- Escapa, I.F., Morales, V., Martino, V.P., Pollet, E., Avérous, L., García, J.L., Prieto, M.A., 2011. Disruption of  $\beta$ -oxidation pathway in *Pseudomonas putida* KT2442 to produce new functionalized PHAs with thioester groups. *Appl Microbiol Biotechnol* 89, 1583–1598. <https://doi.org/10.1007/s00253-011-3099-4>



- Foong, C.P., Lakshmanan, M., Abe, H., Taylor, T.D., Foong, S.Y., Sudesh, K., 2018. A novel and wide substrate specific polyhydroxyalkanoate (PHA) synthase from unculturable bacteria found in mangrove soil. *J Polym Res* 25, 23. <https://doi.org/10.1007/s10965-017-1403-4>
- Furrer, P., Hany, R., Rentsch, D., Grubelnik, A., Ruth, K., Panke, S., Zinn, M., 2007. Quantitative analysis of bacterial medium-chain-length poly([R]-3-hydroxyalkanoates) by gas chromatography. *Journal of Chromatography A* 1143, 199–206. <https://doi.org/10.1016/j.chroma.2007.01.002>
- Hong, J.-W., Song, H.-S., Moon, Y.-M., Hong, Y.-G., Bhatia, S.K., Jung, H.-R., Choi, T.-R., Yang, S., Park, H.-Y., Choi, Y.-K., Yang, Y.-H., 2019. Polyhydroxybutyrate production in halophilic marine bacteria *Vibrio proteolyticus* isolated from the Korean peninsula. *Bioprocess Biosyst Eng* 42, 603–610. <https://doi.org/10.1007/s00449-018-02066-6>
- Imhoff, J.F., Labes, A., Wiese, J., 2011. Bio-mining the microbial treasures of the ocean: New natural products. *Biotechnology Advances, Marine Biotechnology in Europe* 29, 468–482. <https://doi.org/10.1016/j.biotechadv.2011.03.001>
- Korkakaki, E., van Loosdrecht, M.C.M., Kleerebezem, R., 2017. Impact of phosphate limitation on PHA production in a feast-famine process. *Water Res.* 126, 472–480. <https://doi.org/10.1016/j.watres.2017.09.031>
- Korotkova, N., Lidstrom, M.E., 2001. Connection between Poly- $\beta$ -Hydroxybutyrate Biosynthesis and Growth on C1 and C2 Compounds in the Methylophilic Methylobacterium extorquensAM1. *Journal of Bacteriology* 183, 1038–1046. <https://doi.org/10.1128/jb.183.3.1038-1046.2001>
- Laycock, B., Halley, P., Pratt, S., Werker, A., Lant, P., 2014. The chemomechanical properties of microbial polyhydroxyalkanoates. *Progress in Polymer Science, Topical Issue on Biorelated Polymers* 39, 397–442. <https://doi.org/10.1016/j.progpolymsci.2013.06.008>
- Lemechko, P., Le Fellic, M., Bruzaud, S., 2019. Production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) using agro-industrial effluents with tunable proportion of 3-hydroxyvalerate monomer units. *International Journal of Biological Macromolecules* 128, 429–434. <https://doi.org/10.1016/j.ijbiomac.2019.01.170>
- Ma, W.-T., Lin, J.-H., Chen, H.-J., Chen, S.-Y., Shaw, G.-C., 2011. Identification and Characterization of a Novel Class of Extracellular Poly(3-Hydroxybutyrate) Depolymerase from *Bacillus* sp. Strain NRRL B-14911. *Applied and Environmental Microbiology* 77, 7924–7932. <https://doi.org/10.1128/AEM.06069-11>

- McCool, G.J., Cannon, M.C., 2001. PhaC and PhaR are required for polyhydroxyalkanoic acid synthase activity in *Bacillus megaterium*. *J Bacteriol* 183, 4235–4243. <https://doi.org/10.1128/JB.183.14.4235-4243.2001>
- Odobel, C., Dussud, C., Philip, L., Derippe, G., Lauters, M., Eyheraguibel, B., Burgaud, G., Ter Halle, A., Meistertzheim, A.-L., Bruzard, S., Barbe, V., Ghiglione, J.-F., 2021. Bacterial Abundance, Diversity and Activity During Long-Term Colonization of Non-biodegradable and Biodegradable Plastics in Seawater. *Frontiers in Microbiology* 12. <https://doi.org/10.3389/fmicb.2021.734782>
- Pérez-Rivero, C., Hernandez-Raquet, G., 2017. Polyhydroxyalkanoates : une alternative ‘bio’ aux plastiques traditionnels. *Innovations Agronomiques* 58, 99–112.
- Prieto, A., Escapa, I.F., Martínez, V., Dinjaski, N., Herencias, C., de la Peña, F., Tarazona, N., Revelles, O., 2016. A holistic view of polyhydroxyalkanoate metabolism in *Pseudomonas putida*. *Environmental Microbiology* 18, 341–357. <https://doi.org/10.1111/1462-2920.12760>
- Ray, S., Kalia, V.C., 2017. Polyhydroxyalkanoate Production and Degradation Patterns in *Bacillus* Species. *Indian J Microbiol* 57, 387–392. <https://doi.org/10.1007/s12088-017-0676-y>
- Rehm, B.H.A., 2003. Polyester synthases: natural catalysts for plastics. *Biochemical Journal* 376, 15–33. <https://doi.org/10.1042/bj20031254>
- Riis, V., Mai, W., 1988. Gas chromatographic determination of poly- $\beta$ -hydroxybutyric acid in microbial biomass after hydrochloric acid propanolysis. *Journal of Chromatography A* 445, 285–289. [https://doi.org/10.1016/S0021-9673\(01\)84535-0](https://doi.org/10.1016/S0021-9673(01)84535-0)
- Satoh, Y., Minamoto, N., Tajima, K., Munekata, M., 2002. Polyhydroxyalkanoate synthase from *Bacillus* sp. INT005 is composed of PhaC and PhaR. *J Biosci Bioeng* 94, 343–350. <https://doi.org/10.1263/jbb.94.343>
- Sinha, R.K., Krishnan, K.P., Kurian, P.J., 2021. Complete genome sequence of *Alteromonas pelagimontana* 5.12T, a marine exopolysaccharide-producing bacterium isolated from hydrothermally influenced deep-sea sediment of eastern Southwest Indian Ridge. *Marine Genomics* 55, 100804. <https://doi.org/10.1016/j.margen.2020.100804>
- Steinbüchel, A., Valentin, H.E., 1995. Diversity of bacterial polyhydroxyalkanoic acids. *FEMS Microbiology Letters* 128, 219–228. <https://doi.org/10.1111/j.1574-6968.1995.tb07528.x>
- Stubbe, J., Tian, J., 2003. Polyhydroxyalkanoate (PHA) homeostasis: the role of the PHA synthase. *Natural Product Reports* 20, 445–457. <https://doi.org/10.1039/B209687K>
- Sun, Z., Ramsay, J.A., Guay, M., Ramsay, B., 2007. Increasing the yield of *MCL*-PHA from nonanoic acid by co-feeding glucose during the PHA accumulation stage in two-stage fed-batch fermentations of *Pseudomonas putida* KT2440. *Journal of Biotechnology, International*

- Symposium on Biological Polyesters 2006 132, 280–282.  
<https://doi.org/10.1016/j.jbiotec.2007.02.023>
- Thomas, T., Elain, A., Bazire, A., Bruzard, S., 2019. Complete genome sequence of the halophilic PHA-producing bacterium *Halomonas* sp. SF2003: insights into its biotechnological potential. *World J Microbiol Biotechnol* 35, 50. <https://doi.org/10.1007/s11274-019-2627-8>
- Tsuge, T., Hyakutake, M., Mizuno, K., 2015. Class IV polyhydroxyalkanoate (PHA) synthases and PHA-producing *Bacillus*. *Appl Microbiol Biotechnol* 99, 6231–6240. <https://doi.org/10.1007/s00253-015-6777-9>
- Wecker, P., Moppert, X., Simon-Colin, C., Costa, B., Berteaux-Lecellier, V., 2015. Discovery of a *mcl*-PHA with unexpected biotechnical properties: the marine environment of French Polynesia as a source for PHA-producing bacteria. *AMB Express* 5, 74. <https://doi.org/10.1186/s13568-015-0163-y>
- Xie, Y., Noda, I., Akpalu, Y.A., 2008. Influence of cooling rate on the thermal behavior and solid-state morphologies of polyhydroxyalkanoates. *Journal of Applied Polymer Science* 109, 2259–2268. <https://doi.org/10.1002/app.28278>
- Yin, J., Chen, J.-C., Wu, Q., Chen, G.-Q., 2015. Halophiles, coming stars for industrial biotechnology. *Biotechnol Adv* 33, 1433–1442. <https://doi.org/10.1016/j.biotechadv.2014.10.008>
- Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A., 2013. Life in the “Plastisphere”: Microbial Communities on Plastic Marine Debris. *Environ. Sci. Technol.* 47, 7137–7146. <https://doi.org/10.1021/es401288x>
- Zher Neoh, S., Fey Chek, M., Tiang Tan, H., Linares-Pastén, J.A., Nandakumar, A., Hakoshima, T., Sudesh, K., 2022. Polyhydroxyalkanoate synthase (PhaC): The key enzyme for biopolyester synthesis. *Current Research in Biotechnology* 4, 87–101. <https://doi.org/10.1016/j.crbiot.2022.01.002>
- Zhila, N.O., Sapozhnikova, K.Y., Kiselev, E.G., Vasiliev, A.D., Nemtsev, I.V., Shishatskaya, E.I., Volova, T.G., 2021. Properties of Degradable Polyhydroxyalkanoates (PHAs) Synthesized by a New Strain, *Cupriavidus necator* IBP/SFU-1, from Various Carbon Sources. *Polymers* 13, 3142. <https://doi.org/10.3390/polym13183142>

# Chapitre 5 : Conclusion et perspectives

L'originalité de ce travail de thèse a été de réaliser des travaux multidisciplinaires abordant la science des polymères, la biotechnologie, la microbiologie, la biologie moléculaire et cellulaire. La majorité de ces travaux a été réalisée dans les laboratoires de mes deux directeurs de thèse, l'IRDL à Lorient et le LOMIC à Banyuls, où j'ai passé la moitié du temps. Ce travail a pu être réalisé par des collaborations étroites avec les laboratoires Genoscope, ICCF et Plastic@Sea.

Dans ce dernier chapitre, des conclusions et perspectives vont être données pour les 3 points essentiels de cette thèse qui ont permis :

- d'étudier les capacités de biodégradation en milieu naturel marin de *scl*- et *mcl*-PHA produits à façon et de caractériser les communautés bactériennes associées,
- de décrire les mécanismes moléculaires de biodégradation à travers la synthèse *in vitro* et la caractérisation d'une nouvelle enzyme spécialisée dans la dépolymérisation de *scl*-PHA,
- de mettre en évidence la concomitance entre la production et la dégradation de PHA à l'échelle de l'individu via la caractérisation d'un nouveau potentiel de production de *scl*- et *mcl*-PHA chez la souche *A. plasticoclasticus*.

## 1. Etude de la biodégradation des *scl*- et *mcl*-PHA en milieu marin

Une approche novatrice a été mise en place afin d'étudier la biodégradation de plusieurs PHA en mimant autant que possible le milieu naturel marin. Les PHA sont une famille de polymères aux propriétés physico-chimiques variées qui dépendent du substrat utilisé pour leur production et du processus de production utilisés, donnant lieu à des PHA de différents types (*scl*- et *mcl*-PHA). Jusqu'à présent, leur biodégradation en milieu marin naturel avait uniquement été étudiée sur quelques *scl*-PHA (Deroiné et al., 2015, 2014; Volova et al., 2011, 2010). De plus, les techniques utilisées pour tester leur biodégradabilité se limitaient le plus souvent à des observations visuelles ou de pertes de poids et à des tests de respirométrie. D'autre part, les études des microorganismes qui colonisent les PHA en milieu naturel et qui sont susceptibles de les dégrader se limitent à la comparaison d'un PHB ou PHBHV par rapport à d'autres polymères biosourcés ou biodégradables (Odobel et al., 2021; Pinnell and Turner, 2019). Une étude holistique a donc pu être mise en place pour analyser la biodégradation d'une famille de PHA aux compositions et propriétés éclectiques dans les mêmes conditions expérimentales. Les objectifs de cette étude étaient de faire un lien entre

les propriétés intrinsèques des PHA, leur capacité de biodégradation en milieu marin et d'analyser les communautés bactériennes associées.

La production de 3 *scl*- et 3 *mcl*-PHA à partir de deux souches bactériennes et différentes sources de carbone a permis l'élaboration d'une palette de PHA aux propriétés physico-chimiques variées (composition, propriétés thermiques, hydrophobicité et masse molaire).

Après analyse de leur biodégradation en milieu marin, la première conclusion a été que les *scl*-PHA présentent une biodégradation plus élevée malgré une masse molaire, une cristallinité plus élevées que les *mcl*-PHA, ces facteurs dits extrinsèques étant en général plutôt favorables à une certaine stabilité. Ces résultats, a priori surprenants, suggèrent que la composition chimique du PHA, et plus particulièrement la structure de la chaîne latérale du polymère (chaîne courte ou moyenne), joue un rôle clé dans les processus de biodégradation en milieu naturel. En effet, aucune différence significative au sein d'une même catégorie de PHA (*scl*- ou *mcl*-) n'a pu être mise en évidence à cette échelle. Notre hypothèse pour expliquer un tel impact de la structure de la chaîne latérale sur la biodégradation résulterait de la spécificité de la PHA dépolymérase extracellulaire vis-à-vis des *scl*- ou des *mcl*-PHA. En effet, il a été montré que l'activité du domaine catalytique était différente entre les *scl*- et les *mcl*-PHA dépolymérases, rendant la *mcl*-PHA dépolymérase inefficace sur les *scl*-PHA, et inversement (Kim et al., 2000). D'autre part, la longueur de la chaîne carbonée des *mcl*-PHA (qui présente un encombrement stérique supérieur à celui des *scl*-PHA) pourrait inhiber la dégradation enzymatique en empêchant le domaine catalytique d'accéder aux liaisons esters, en raison de la gêne stérique provoquée par la taille des groupes latéraux (Numata et al., 2009). Des travaux complémentaires seront nécessaires pour valider cette hypothèse, et notamment en allant plus loin sur l'analyse des dépolymérases externes. Comme nous l'avons vu dans le chapitre 3 de cette thèse, il nous a été possible de mieux caractériser les dépolymérases externes impliquées dans la dégradation des *scl*-PHA. Néanmoins, ce travail réalisé à l'échelle de souches isolées ne permet pas une extrapolation au milieu naturel complexe. Pour avoir une meilleure vision des dépolymérases externes en milieu naturel, nous proposons de poursuivre ces travaux en utilisant un couplage entre un marquage isotopique et des analyses métagénomiques. Des travaux préliminaires que j'ai pu initier lors de cette thèse, mais qui n'ont pas été présentés, ont consisté à utiliser des *scl*- et des *mcl*-PHA marqués par des isotopes stables du carbone. Du glucose ou de l'acide nonanoïque marqués ont été incorporés pour permettre la production de  $^{13}\text{C}$ -*scl*-PHA ( $^{13}\text{C}$ -PHB) et de  $^{13}\text{C}$ -*mcl*-PHA ( $^{13}\text{C}$ -PHN). La technique de DNA-stable isotope probing permet de séparer les ADN des cellules marquées au  $^{13}\text{C}$  (qui ont métabolisé le substrat marqué) de celles marquées au  $^{12}\text{C}$  (qui n'ont pas métabolisé le substrat marqué) au sein d'une communauté complexe (Dunford and Neufeld, 2010). Nos résultats

préliminaires montrent que cette technique est utilisable pour une analyse métagénomique à partir de *scl*-PHA marqués au  $^{13}\text{C}$ . Le temps imparti à cette thèse ne m'a pas permis de reproduire cette expérience pour une analyse métagénomique à partir de *mcl*-PHA marqués au  $^{13}\text{C}$ . La comparaison des données métagénomiques des fractions issues des *scl*- et des *mcl*-PHA marquées aux  $^{13}\text{C}$  permettraient de différencier la spécificité des dépolymérase externes pour chacun des types de PHA en milieu naturel.

Un autre aspect de ce travail a concerné la diversité bactérienne associée à la biodégradation des PHA. Comme nous l'avons souligné précédemment, il s'agit de la première étude à réaliser un tel suivi en milieu marin. Nous avons pu observer une plus faible diversité bactérienne associée à la dégradation rapide des *scl*-PHA par rapport aux *mcl*-PHA, suggérant une plus grande spécialisation des communautés associées à la fonction de biodégradation dans le cas des *scl*-PHA. En particulier, nous avons identifié deux taxa pour leur contribution importante dans la biodégradation des *scl*-PHA : *Marinobacter sp.* connu pour ses capacités de dégradation du PHB et du PHBHV (Kasuya et al., 2000; Martínez-Tobón et al., 2018) et *Cobetia sp.* connu pour ses capacités de production de *scl*-PHA (Christensen et al., 2021; Moriya et al., 2020). Bien qu'une diversité plus importante soit présente sur les *mcl*-PHA, le taxa appartenant à *Pseudomonas sp.* connu pour produire et dégrader les PHA (Prieto et al., 2016; Schirmer et al., 1993; Schirmer and Jendrossek, 1994) ainsi que des Flavobacteriaceae ont montré une certaine contribution sur ce type de polymère. L'identification de ces espèces clés dans le processus naturel de biodégradation des PHA en milieu marin permettra de justifier de poursuivre des travaux sur le métabolisme de dégradation des PHA en ciblant ces espèces en particulier.

Grace à notre capacité originale de tester en parallèle la biodégradabilité de plusieurs polymères dans les mêmes conditions expérimentales (voir la partie *Materials and Methods* du chapitre 2), cette étude nous a permis de présenter des résultats originaux pour corréler de manière globale les propriétés intrinsèques relatives à la famille des PHA et leur biodégradation en milieu marin naturel. Si une distinction nette a pu être établie entre la biodégradabilité des *scl*-PHA et des *mcl*-PHA, nos travaux réalisés sur des périodes courtes n'ont pas permis d'approfondir les facteurs clés de la biodégradation des *mcl*-PHA. Nous proposons que des travaux complémentaires soient réalisés dans les mêmes conditions expérimentales que dans le chapitre 2 mais sur des périodes de temps plus longues. Ces travaux permettraient de mieux comprendre les conditions de biodégradation des *mcl*-PHA en milieu marin.

## 2. Etude de la dégradation *in vitro* des PHA par des PHA depolymérase

Afin de mieux comprendre les mécanismes moléculaires impliqués dans la dégradation des PHA, une approche *in vitro* a été privilégiée par la synthèse et la caractérisation d'enzymes. Cette approche permet une analyse fine des mécanismes de dégradation, tout en s'affranchissant de l'influence des facteurs environnementaux et du biofilm. Ce travail a été rendu possible grâce à l'implication de plusieurs laboratoires ayant de fortes compétences en sciences des polymères, microbiologie, génétique, biologie moléculaire, biochimie et chimie analytique. L'étude à l'échelle moléculaire de la dégradation des PHA par voie enzymatique a permis la caractérisation d'une nouvelle *scl*-PHA dépolymérase (dPhaZ-Apla) en lien avec la récente découverte de la bactérie marine *Alteromonas plasticoclasticus* MED1.

Ce travail présente la production et la caractérisation de la protéine dPhaZ-Apla à partir de la bactérie marine *Alteromonas plasticoclasticus* MED1. La dPhaZ-Apla présente un site catalytique de type I et deux SBD similaire à l'enzyme d'*Alcaligenes faecalis* AE122 (Kita et al., 1997). L'activité de cette nouvelle enzyme a montré des taux de dégradation inférieurs à ceux des autres enzymes dPhaZ<sub>SCL</sub>, c'est-à-dire les enzymes dPhaZ-Rpic et dPhaZ-Apla de *Ralstonia pickettii* T1 et *Alcaligenes faecalis* AE122, respectivement, via diverses méthodes : perte de poids du matériel, dosage des oligomères par résonance magnétique nucléaire et microscopie électronique à balayage.

La dépolymérase dPhaZ-Apla a montré une spécificité similaire à celle des enzymes dPhaZ-Afae et dPhaZ-Rpic avec une dégradation plus élevée du PHBHV (plus amorphe) que du PHB (plus cristallin). L'ensemble des enzymes ont également montré une coupure aléatoire de la chaîne polymère, sans distinction entre les motifs HB ou HV. Ces résultats indiquent l'affinité des enzymes pour des surfaces aux régions amorphes plus élevés. Aux vues de ces résultats, nous proposons de poursuivre ces travaux en se focalisant sur l'impact des propriétés de surface sur l'activité des dPhaZ<sub>SCL</sub>. Des travaux préliminaires de ma thèse qui n'ont pas été discutés dans ce manuscrit ont consisté à évaluer les conséquences de l'attaque enzymatique sur la structure des polymères en utilisant la microscopie à force atomique. Par manque de temps, ces travaux n'ont pas pu être concluants et nécessitent un important travail sur la réalisation de films ultra minces (~100 nm) de PHA. Cette approche permettrait d'étudier plus finement les spécificités des différentes enzymes à l'échelle macro- et micro-moléculaire.

D'autres études seront également nécessaires pour optimiser les conditions expérimentales de l'utilisation de l'enzyme dPhaZ-Apla, afin de mieux déterminer son potentiel d'activité. Par exemple, les activités de l'enzyme à différentes conditions de pH ou de température restent à tester. De plus, la

pureté de l'enzyme dPhaZ-Apla pourrait être améliorée afin de standardiser sa concentration lors de la mise en place des réactions enzymatiques. D'autre part, le travail présenté sur les PHA dépolymérase dans cette thèse s'est concentré sur les *scl*-PHA dépolymérase. Durant cette thèse, nous avons également initié l'étude de la dégradation *in vitro* de *mcl*-PHA par des *mcl*-PHA dépolymérase. Deux enzymes ont été produites : dPhaZ-Pflu provenant de *Pseudomonas fluorescens* *GK13* (Schirmer and Jendrossek, 1994) et dPhaZ<sub>mcl</sub> identifiée chez *Alteromonas plasticoclasticus* *MEDI*. L'étude de ces enzymes permettrait de mieux comprendre les mécanismes moléculaires de dégradation de *mcl*-PHA, qui semblent plus récalcitrant que ceux impliqués dans la dégradation de *scl*-PHA.

### 3. Production concomitante de *scl*- et *mcl*-PHA par la bactérie marine *Alteromonas plasticoclasticus* *MEDI*

Lors de l'isolement de la nouvelle espèce bactérienne *Alteromonas plasticoclasticus* *MEDI*, isolée d'un biofilm se développant sur un PHA en conditions naturelles, ses capacités de dégradation du PHB et du PHBHV y étaient initialement associées. L'analyse plus poussée de son génome avait également montré la présence de gènes impliqués dans la synthèse de PHA. Les travaux de cette thèse ont permis de confirmer les activités des PHA synthèses identifiées dans son génome.

Nous avons montré les capacités de cette souche à produire du PHB et du PHN à partir de glucose et d'acide nonanoïque, respectivement. L'analyse des voies métaboliques suggère l'utilisation de la glycolyse pour la production du PHB et de la  $\beta$ -oxydation pour la production du PHN. Nos résultats montrent que la souche *Alteromonas plasticoclasticus* *MEDI* possède deux opérons séparés impliqués chacun dans la production de *scl*- et *mcl*-PHA.

Les propriétés associées au PHA produit dans cette étude sont conformes à celles des PHA produits par la souche productrice de *scl*-PHA *Halomonas* *sp.* *SF2003* et par la souche productrice de *mcl*-PHA *Pseudomonas putida* *KT2440*. Cependant, les rendements sont plus faibles que ces deux dernières bactéries bien étudiées (Lemechko et al., 2019; Sun et al., 2009).

Un substrat de croissance ou d'accumulation plus approprié peut être utilisé pour améliorer la teneur en PHA dans les cellules d'*Alteromonas plasticoclasticus* *MEDI*. Le déclenchement d'un stress tel que la limitation en azote ou en phosphore est également une méthode efficace pour améliorer la quantité de PHA dans les cultures (Ahn et al., 2015; Korkakaki et al., 2017). Bien que ces paramètres de processus, comme d'autres substrats de croissance et d'accumulation n'aient pas été explorés dans cette étude, ils constituent les principales perspectives pour améliorer les teneurs en PHA dans les cultures d'*Alteromonas plasticoclasticus* *MEDI*.



De manière plus générale, nous avons montré que notre stratégie de sélection de souche bactérienne à partir d'un biofilm naturel marin avait porté ses fruits pour la découverte d'une nouvelle souche aux propriétés originales *Alteromonas plasticoclasticus* MED1. Cette souche est la première souche à être décrite comme accumulant à la fois des *scl*- et *mcl*-PHA de façon concomitante et la première souche marine à produire du *mcl*-PHA par la voie de la  $\beta$ -oxydation à partir d'un acide gras. C'est un résultat extrêmement prometteur qui ouvre la voie à de multiples pistes en termes de conception de PHA présentant des structures et des propriétés inconnues à ce jour. Nous proposons que l'utilisation de la plastisphère soit un nouveau réservoir pour isoler de nouvelles espèces qui pourraient avoir un intérêt pour les biotechnologies, notamment en lien avec la production de PHA.

L'approche pluridisciplinaire de cette thèse en lien avec les différents laboratoires partenaires nous a permis de trouver des résultats originaux qui n'auraient pas pu être mis en évidence individuellement. Ces travaux contribuent à une amélioration des connaissances fondamentales des processus de production et de dégradation des PHA en milieu naturel. Ils ont également un intérêt en biotechnologies, puisqu'ils ouvrent de nouvelles voies de recherche pour la production de PHA à façon, avec des propriétés physico-chimiques variées et des taux de biodégradation adaptés aux cahiers des charges du matériau.

## Références

- Ahn, J., Jho, E.H., Nam, K., Ahn, J., Jho, E.H., Nam, K., 2015. Effect of C/N ratio on polyhydroxyalkanoates (PHA) accumulation by *Cupriavidus necator* and its implication on the use of rice straw hydrolysates. *Environmental Engineering Research* 20, 246–253. <https://doi.org/10.4491/eer.2015.055>
- Christensen, M., Jablonski, P., Altermark, B., Irgum, K., Hansen, H., 2021. High natural PHA production from acetate in *Cobetia* sp. MC34 and *Cobetia marina* DSM 4741T and in silico analyses of the genus specific PhaC2 polymerase variant. *Microbial Cell Factories* 20, 225. <https://doi.org/10.1186/s12934-021-01713-0>
- Deroiné, M., César, G., Le Duigou, A., Davies, P., Bruzaud, S., 2015. Natural Degradation and Biodegradation of Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) in Liquid and Solid Marine Environments. *J Polym Environ* 23, 493–505. <https://doi.org/10.1007/s10924-015-0736-5>
- Deroiné, M., Le Duigou, A., Corre, Y.-M., Le Gac, P.-Y., Davies, P., César, G., Bruzaud, S., 2014. Seawater accelerated ageing of poly(3-hydroxybutyrate-co-3-hydroxyvalerate). *Polymer Degradation and Stability* 105, 237–247. <https://doi.org/10.1016/j.polymdegradstab.2014.04.026>
- Dunford, E.A., Neufeld, J.D., 2010. DNA stable-isotope probing (DNA-SIP). *J Vis Exp* 2027. <https://doi.org/10.3791/2027>
- Kasuya, K., Mitomo, H., Nakahara, M., Akiba, A., Kudo, T., Doi, Y., 2000. Identification of a Marine Benthic P(3HB)-Degrading Bacterium Isolate and Characterization of Its P(3HB) Depolymerase. *Biomacromolecules* 1, 194–201. <https://doi.org/10.1021/bm9900186>
- Kita, K., Mashiba, S., Nagita, M., Ishimaru, K., Okamoto, K., Yanase, H., Kato, N., 1997. Cloning of poly(3-hydroxybutyrate) depolymerase from a marine bacterium, *Alcaligenes faecalis* AE122, and characterization of its gene product. *Biochim Biophys Acta* 1352, 113–122. [https://doi.org/10.1016/s0167-4781\(97\)00011-0](https://doi.org/10.1016/s0167-4781(97)00011-0)
- Korkakaki, E., van Loosdrecht, M.C.M., Kleerebezem, R., 2017. Impact of phosphate limitation on PHA production in a feast-famine process. *Water Res.* 126, 472–480. <https://doi.org/10.1016/j.watres.2017.09.031>
- Lemechko, P., Le Fellic, M., Bruzaud, S., 2019. Production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) using agro-industrial effluents with tunable proportion of 3-hydroxyvalerate monomer units. *International Journal of Biological Macromolecules* 128, 429–434. <https://doi.org/10.1016/j.ijbiomac.2019.01.170>
- Martínez-Tobón, D.I., Gul, M., Elias, A.L., Sauvageau, D., 2018. Polyhydroxybutyrate (PHB) biodegradation using bacterial strains with demonstrated and predicted PHB depolymerase activity. *Appl Microbiol Biotechnol* 102, 8049–8067. <https://doi.org/10.1007/s00253-018-9153-8>
- Moriya, H., Takita, Y., Matsumoto, A., Yamahata, Y., Nishimukai, M., Miyazaki, M., Shimoi, H., Kawai, S.-J., Yamada, M., 2020. *Cobetia* sp. Bacteria, Which Are Capable of Utilizing Alginate or Waste *Laminaria* sp. for Poly(3-Hydroxybutyrate) Synthesis, Isolated From a Marine Environment. *Frontiers in Bioengineering and Biotechnology* 8. <https://doi.org/10.3389/fbioe.2020.00974>
- Odobel, C., Dussud, C., Philip, L., Derippe, G., Lauters, M., Eyheraguibel, B., Burgaud, G., Ter Halle, A., Meistertzheim, A.-L., Bruzaud, S., Barbe, V., Ghiglione, J.-F., 2021. Bacterial Abundance, Diversity and Activity During Long-Term Colonization of Non-biodegradable and Biodegradable Plastics in Seawater. *Frontiers in Microbiology* 12. <https://doi.org/10.3389/fmicb.2021.734782>

- Pinnell, L.J., Turner, J.W., 2019. Shotgun Metagenomics Reveals the Benthic Microbial Community Response to Plastic and Bioplastic in a Coastal Marine Environment. *Frontiers in Microbiology* 10.
- Prieto, A., Escapa, I.F., Martínez, V., Dinjaski, N., Herencias, C., de la Peña, F., Tarazona, N., Revelles, O., 2016. A holistic view of polyhydroxyalkanoate metabolism in *Pseudomonas putida*. *Environ. Microbiol.* 18, 341–357. <https://doi.org/10.1111/1462-2920.12760>
- Schirmer, A., Jendrossek, D., 1994. Molecular characterization of the extracellular poly(3-hydroxyoctanoic acid) [P(3HO)] depolymerase gene of *Pseudomonas fluorescens* GK13 and of its gene product. *J Bacteriol* 176, 7065–7073.
- Schirmer, A., Jendrossek, D., Schlegel, H.G., 1993. Degradation of poly(3-hydroxyoctanoic acid) [P(3HO)] by bacteria: purification and properties of a P(3HO) depolymerase from *Pseudomonas fluorescens* GK13. *Appl. Environ. Microbiol.* 59, 1220–1227.
- Sun, Z., Ramsay, J., Guay, M., Ramsay, B., 2009. Enhanced yield of medium-chain-length polyhydroxyalkanoates from nonanoic acid by co-feeding glucose in carbon-limited, fed-batch culture. *Journal of Biotechnology* 143, 262–267. <https://doi.org/10.1016/j.jbiotec.2009.07.014>
- Volova, T.G., Boyandin, A.N., Vasil'ev, A.D., Karpov, V.A., Kozhevnikov, I.V., Prudnikova, S.V., Rudnev, V.P., Xuân, B.B., Dũng, V.V., Gitel'zon, I.I., 2011. Biodegradation of polyhydroxyalkanoates (PHAs) in the South China Sea and identification of PHA-degrading bacteria. *Microbiology* 80, 252–260. <https://doi.org/10.1134/S0026261711020184>
- Volova, T.G., Boyandin, A.N., Vasiliev, A.D., Karpov, V.A., Prudnikova, S.V., Mishukova, O.V., Boyarskikh, U.A., Filipenko, M.L., Rudnev, V.P., Bá Xuân, B., Việt Dũng, V., Gitelson, I.I., 2010. Biodegradation of polyhydroxyalkanoates (PHAs) in tropical coastal waters and identification of PHA-degrading bacteria. *Polymer Degradation and Stability* 95, 2350–2359. <https://doi.org/10.1016/j.polymdegradstab.2010.08.023>

# Annexe F

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## **Bioplastic degradation and assimilation processes by a novel bacteria isolated from the marine plastisphere**

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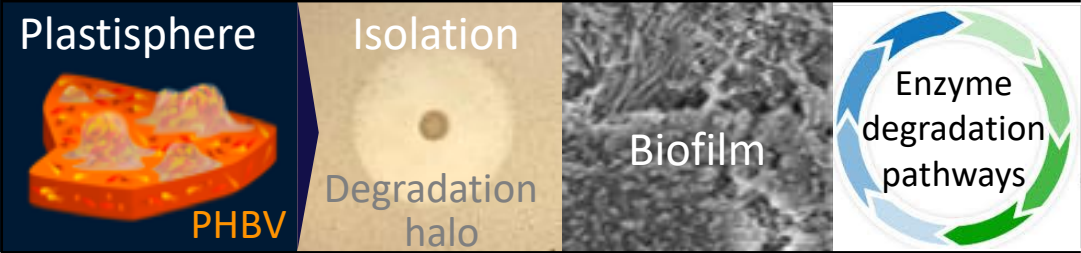
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**Keywords:** Polyhydroxyalkanoate (PHA), plastic biodegradation, biofilm, glyoxylate shunt, depolymerase

**Graphical abstract:**



## Highlights:

- Novel bacteria named *Alteromonas plasticoclasticus* isolated from the marine plastisphere
- *A. plasticoclasticus* is a new marine PHBV-degrader forming biofilms
- First description of the entire enzymatic pathways of PHBV degradation by a marine bacteria

**Abstract:** Biosourced and biodegradable plastics offer a promising solution to reduce environmental impacts of plastics for specific applications. Here, we report a novel bacteria named *Alteromonas plasticoclasticus* isolated from the marine plastisphere that form biofilms on the biosourced and biodegradable Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) films. Degradation halo, weight loss, oxygen consumption and bacterial heterotrophic activity revealed its ability to degrade PHBV as sole carbon source. Genomic and transcriptomic analysis permitted the first description of the entire enzymatic pathways used by a marine bacteria to degrade PHBV. A gene cluster coding for key enzymes involved in the external depolymerization (ePHAZ) was discovered, together with the cascade of genes involved in the complete mineralization of PHBV. Interestingly, this bacteria uses the glyoxylate shunt rather than the complete TCA cycle to regulate carbon fluxes during PHBV degradation. This results show the potential of the natural plastisphere to discover new bacteria with promising metabolic capabilities.

## 1.Introduction :

Plastic distribution in both the terrestrial, atmospheric and aquatic fields suggests that they are a key geological indicator of the Anthropocene (Avelar Santos et al, 2022). Conventional plastics derived from petrochemicals are generally categorized as non-biodegradable and accumulate in all realms, causing irreversible damage to fauna and flora (Charlton-Howard et al. 2023, Johnston et al. 2023). As a partial solution, it has been proposed to manufacture plastics that would be both bio-based, i.e., made from renewable resources such as agricultural waste, and biodegradable (totally transformed in biomass and CO<sub>2</sub>) in a given environment (compost, soil, water) over a reasonable amount of time (weeks to months). Among the bio-based and biodegradable polymers, polyhydroxyalkanoates (PHA) are considered as a promising alternative to petroleum-based or non-biodegradable polymers. Mainly but not only from bacterial origin, PHA constitute a large family and display a wide range of chemical compositions and properties according to the producing strain, the source of carbon used for feeding and the fermentation process. Among them, poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) has been shown to exhibits excellent marine

biodegradability as compared to the polylactic acid (PLA), which is the current leading ‘biodegradable’ polymer in the plastics market but showed no sign of degradation in the marine environment (Cheng et al. 2022, Royer et al. 2023). PHBV is a very promising polymer that has great potential for selected applications with respect to their use and end of life, and to play a significant role in the circular economy concept (Paul-Pont et al. 2023). For example, fishery gears and aquaculture equipment that are likely to degrade over time and/or be lost at sea could have less long-term impacts on marine fauna (e.g., entanglement, ghost fishing, wounds) if they were made of marine biodegradable materials, such as PHBV (Ibrahim et al. 2021). Other applications include the plastic particles and water-soluble polymers used in care products (e.g., cosmetics, detergents) or the microfibers used in textiles (Sahlan et al. 2020; Volant et al. 2021). Understanding of the processes of PHBV biodegradation by bacteria is an important challenge to evaluate its driving forces in the marine environment.

Dissimilar microorganisms were shown to form a mature biofilm (called ‘plastisphere’) on PHBV as compared to other polymers such as PLA or polyethylene (PE) in natural marine conditions (Odobel et al. 2021). Within the marine plastisphere, potential PHBV-degraders were identified, such as *Marinobacter* sp. and *Alteromonas* sp. (Jacquin et al. 2021), but the biodegradation processes was not studied for these species. Recently, a new bacteria belonging to the genus *Nocardioides* was isolated from the plastisphere with P(3HB)-degrading capabilities (Suzuki et al. 2020), thus reinforcing the interest of using the plastisphere as a new habitat to isolate PHA-degrading bacteria. PHA are degraded by a large set of terrestrial or aquatic microorganisms (Jacquin et al. 2019) thanks to a key enzyme called depolymerase, PhaZ (Klingbeil, Reiner and Jendrossek 1996; Jendrossek and Handrick 2002; Jendrossek 2005). Secreted depolymerases (ePhaZ) degrade extracellular amorphous or crystalline PHA (called denatured PHA, dPHA) (Jendrossek and Handrick 2002). These enzymes are carboxylesterases belonging to the  $\alpha / \beta$  hydrolase family members. The comparison of several PHA depolymerases revealed a common catalytic triad (histidine - serine - aspartic acid) (Jendrossek 2007). To date, several external depolymerases (ePhaZ) have been identified from various isolated strains (DED database, Knoll et al., 2009, Viljakainen and Hug 2021), but very few were originating from the biofilm living on plastic in the environment (the so-called “plastisphere”; Zettler et al. 2013). Moreover, the entire metabolic pathways involved in the degradation of PHA remains unexplored. In particular, there is a growing interest in describing the enzymes involved in the plastic biodegradation that offer future perspectives for industrial applications (Tournier et al., 2023).

In this study, we used a PHBV-selection pressure on the marine plastisphere to discover a new bacteria with PHBV biodegradation capabilities. We tested a large set of complementary approaches (degradation halo, weight loss, scanning electron microscopy, oxygen and bacterial heterotrophic

activity measurements) to characterize its PHBV biodegradation capabilities. Then, coupled Illumina and Nanopore sequencing strategies were used for full genome sequencing and transcriptomic analysis in order to describe for the first time the cascade of genes expression controlling the PHBV degradation and assimilation.

## 2. Materials and Methods

### 2.1. A three steps protocol for the selection of a new PHA-degrading bacteria

We used an original three step protocol to isolate a new strain able to biodegrade PHBV as sole carbon source and energy. The first step consisted of the formation of a mature biofilm in natural seawater, as previously described (Odobel et al. 2021). Briefly, several pieces of PHBV films (around 5 cm<sup>2</sup> each) were incubated for a period of 2-months in 1.8 L aquarium (Sodispan, Spain) with direct circulation to the sea in the Banyuls-sur-mer Bay (close to the SOLA observatory station of the Laboratoire Arago, NW Mediterranean Sea). A flow rate of 50 ml min<sup>-1</sup> was chosen to ensure a sufficient renewal of natural bacteria (every 30 min) and the surface outlet water was filtered at 50 µm. Second, three pieces of PHBV films were transferred under sterile conditions to 12 mL closed Exetainer tube (Interchim, France) containing 3 mL of minimum medium (MM) (see Suppl. 1 for medium composition). Vials were incubated in the dark at 18°C under agitation at 110 rpm (orbital agitator) for a period of 2 months. Controls with MM only in the vials were incubated in the same conditions. Continuous measurement of dissolved oxygen (every hour during the 2 months) was carried out by a Fiber Optic Oxygen Meter box (Fibox 4, Presens) after fitting in the vials an oxygen sensor (Presens, SP-PSt3-YAU) connected to a luminescent optical fiber. Third, the cells growing on PHBV were detached by using three cycles of soft sonication and vortexing, which was previously shown not to alter the bacterial activities (Dussud et al. 2018). Successive dilutions were carried out (10<sup>7</sup> to 10<sup>2</sup> cells/ml) in Marine Broth (MB; Difco) diluted at 1/10 (v/v), left for 4 hours at 18°C and 100 µl of each dilution were spread on Marine Agar (MA; Difco) for 2 days at 18°C. The resulting colonies were transferred onto plates for 10 days at 18°C with MM and noble agar (see Suppl. 1 for medium composition) together with molten PHBV as carbon source. All the colonies showing the presence of a degradation halo were isolated (Rodriguez et al. 2010). The colony labeled *MEDI* and with the largest halo was selected for this study. This colony was further cultured in MB for 24 h at 18°C and stored at -80°C in glycerol (30% V/V).

### 2.2. Characterization of the PHA-degrading capabilities by the *MEDI* strain.



Cells from the *MEDI* strain were pre-cultured in MB and transferred to MM with no other carbon source than the added 10 mM of Glucose (Sigma-Aldrich) for 72 h at 18°C. The cells were rinsed twice with MM to remove all traces of Glucose.

Six triplicate of the same inoculum ( $10^7$  cells ml<sup>-1</sup>) were then incubated (18°C, 300 rpm) in serum flasks (Dutscher) containing 10 ml of MM supplemented or not with a piece (1 cm<sup>2</sup>) of PHBV film or with 10 mM Glucose. The PHBV films were previously sterilized (15 min UV exposure, 48h with ethanol 70% and drying under sterile hood). Oxygen consumption, heterotrophic activity and weight loss were measured in order to confirm the PHBV biodegradation capability of *MEDI* under controlled conditions (temperature, pH, nutrients).

Continuous measurement of dissolved oxygen (every hour) was carried out during the course of the experiment by a Fiber Optic Oxygen Meter box (Fibox 4, Presens) after fitting in the vials an oxygen sensor (Presens, SP-PSt3-YAU) connected to a luminescent optical fiber. In order to maintained dissolved oxygen concentration above 50 µM, the tubes containing the PHBV together with the *MEDI* strain were flushed with air filtered at 0.2 µm under a sterile laminar flow hood on days 5, 11, 16 and 24.

Heterotrophic bacterial activity was measured in triplicate using the <sup>3</sup>H-leucine (specific activity 123.8 Ci mmol<sup>-1</sup>; Perkin Elmer) incorporation into proteins method (final leucine concentrations were 150 nmol/l), as previously described (Conan et al. 2022). Samples were taken after 0, 5, 11, 16, 32 and 40 days for PHBV and for Glucose experiments. The theoretical conversion factor of 1.55 ngC.pmol<sup>-1</sup> of incorporated leucine was used to calculate heterotrophic bacterial production (Simon and Azam, 1989).

Weight loss (in %) was estimated at the beginning (T0) and at the end of the experiment (T40) after rinsing the PHBV films with distilled water and using a precision balance (Precisa 125 A, Swiss quality).

Inorganic nutrients including nitrate (NO<sub>3</sub><sup>-</sup>), nitrite (NO<sub>2</sub><sup>-</sup>), ammonium (NH<sub>4</sub><sup>+</sup>) and phosphate (PO<sub>4</sub><sup>3-</sup>) were quantified at the same sampling points as for heterotrophic bacterial activity (see above) by using a Bran Luebbe III autoanalyzer, as previously described (Sauret et al. 2015). Stability of temperature (company) and pH (company) was measured every two days.

One sample containing PHBV with the strain or not (control) were fixed in 3% Glutaraldehyde after 0, 5, 11, 16, 32 and 40 days, and stored at 4°C until scanning electron microscope observation (Hitachi SEM FEG S-4500 high vacuum), as previously described (Jacquin et al. 2020).

### 2.3. Genomic analysis and taxonomic affiliation.

Genomic DNA was obtained from a pure culture of the *MEDI* strain in MB for 48 hours at 18°C. DNA extraction was performed using phenol-chloroform method, as previously described (Sauret et al. 2016). DNA was quantified by spectrophotometry (Quant-iT™ PicoGreen™ dsDNA Assay Kit, Invitrogen).

The complete DNA sequence of the *MEDI* strain was obtained using two sequencing technologies for short (Illumina) and long reads (Oxford Nanopore Technologies). The Illumina library was prepared using 100 ng of genomic DNA after DNA fragmentation to an average size of 500-600 bp using a Covaris E220. The ends of the DNA fragments were repaired, dATP was added and the adapters (NEXTflex HT Barcodes) were ligated using the NEBNext DNA modules. After being purified twice consecutively with AMPure XP (1 volume), the DNA fragments were amplified by PCR (12 cycles) with the Kapa Hifi Hotstart NGS library amplification kit, and then purified again with AMPure XP (0.7 volume). After being quantified by qPCR, the library was sequenced on a MiSeq device in “paired end” (2x250bp). A total of 1,551,185 (466 Mb) read pairs were obtained and used for assembly. To complete this data set, a first Nanopore library was prepared from 825 ng of genomic DNA according to the “Genomic DNA by Ligation” protocol (SQK-LSK109) recommended by Oxford Nanopore. This library was loaded on a Flongle R9.4.1 flowcell leading to 76,547 sequences (126 Mb) with an N50 of 10,801 bp. To improve these data, a second library was prepared according to the same protocol from 700 ng of genomic DNA and loaded on a MinION R9.4.1 flowcell, leading to 641,816 sequences (1.130 Gb) with an N50 of 14,665 bp. Unicycler software v.0.4.6 (Wick et al. 2017) was run using default options with all 3 datasets. The automatic annotation was performed via the MicroScope platform (<https://mage.genoscope.cns.fr/microscope/home/index.php>). Specific attention has been made to the identification of external depolymerases (ePhaZ). The protein sequences of all the CDSs of the genome were compared using blastp (with parameters: evaluate 1.0, word\_size: 5 and threshold: 10) to the ePhaZ of the DED database (Knoll et al. 2009). Sequences with more than 98% identity from the same organism have been assigned to a single protein.

Taxonomic affiliation of the *MEDI* strain was performed on the purified PCR products (AmpliClean™ Cleanup Kit) using the Sanger sequencing technology with the ProDye™ Terminator Sequencing System (Promega, France), primer 907R (CCGTCAATTCCTTTGAGTTT) and ABI 3130 genetic analyzer (Life Technologies), as previously described (Fagervold et al., 2013). Sequences with >97% identity (NCBI, Blastnt) served to build a database (16 strains belonging to the Alteromonadaceae family). In addition, the 16S rDNA sequence of *Marinobacter hydrocarbonoclasticus* ATCC 49840 has been added to the database since this genus has been already identified on PHBV and hydrophobic organic compounds degradation (Grimaud et al. 2012; Kong et

al. 2018). Clustal Omega software (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) was used to build a phylogenetic tree based on the neighbor-joining without distance corrections method on the 17 strains (reference). The tree data was then visualized using Interactive tree of life (ItoI, <https://itol.embl.de/>, ref). To go further in the classification of the strain and to validate that it belonged to a new species, the Genome Taxonomy Database toolkit (GTDB-Tk) (version 2.1.1) (<https://ecogenomics.github.io/GTDBTk/>, Chaumeil et al. 2020) was performed on the complete genome with the command line options “classify\_wf”.

#### 2.4. Functional annotation from transcriptomic analysis

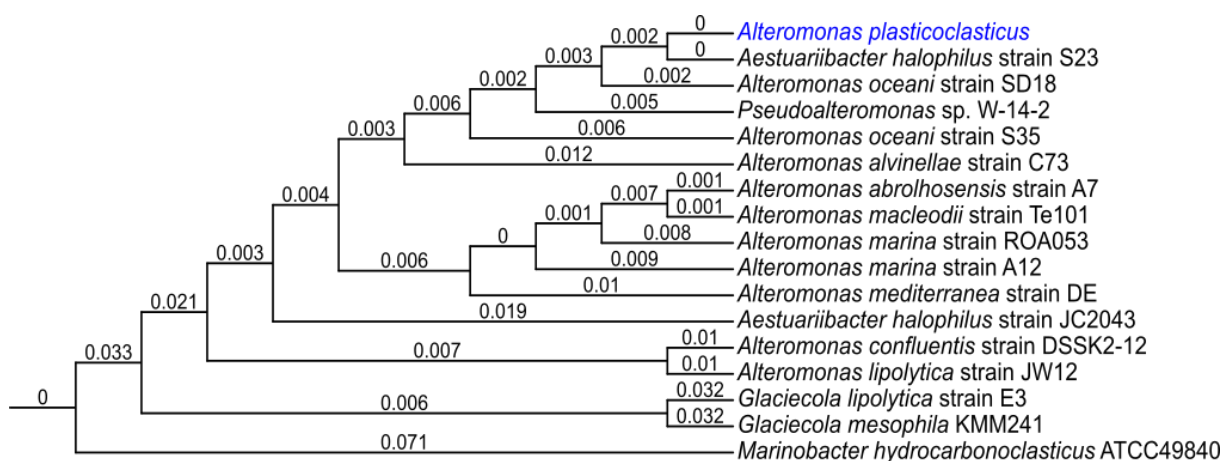
Triplicate RNA from the *MEDI* strain were extracted after 11 days of incubation in MM with PHBV or after 31 hours in MM with Glucose, i.e. corresponding to the highest bacterial activities ( $O_2$  consumption, heterotrophic activity) measured during the course of the experiment. RNA extraction was carried out by using FastPrep beads beating system (twice 15 seconds in TE buffer) for bacterial lysis, followed by phenol-choloroform technique, as previously described (Sauret et al., 2016). RNA were quantified using the Qbit RNAHS kit. A total of 89863741 and 95464415 reads were produced with Illumina sequencing in Glucose or PHBV conditions respectively.

Transcriptome sequencing data were analyzed using the bioinformatics pipeline implemented in the MicroScope platform (Vallenet et al. 2020). Raw reads were first mapped onto the reference genome of the *MEDI* strain (BWA-MEM v.0.7.4) (Li, 2013). An alignment score equal to at least half of the reads was required for a hit to be retained. To lower the false positive discovery rate, the SAMtools (v.0.1.8; Li et al., 2009) were used to extract reliable alignments with a Mapping Quality (MAPQ)  $\geq 1$  from SAM formatted files. The number of reads matching each genomic object (GO) on the reference genome was subsequently computed with the toolset BEDTools (v.2.10.1; Quinlan and Hall 2010). Finally, the R package DESeq2 (v.1.22.2; Love, Huber, and Anders 2014) was used with lfcShrink function to analyze raw counts data and test for differential expression between chosen experimental conditions. For each comparison, differential gene expression was considered significant for a computed p-adjusted  $\leq 0.05$  and for an absolute value of the  $\log_2(\text{fold change}) \geq 0.5$ . The functional analysis of differentially expressed genes (DEG) was perform using the RNA-Seq analysis module in the Experimental Data tab of “Microscope” platform (<https://mage.genoscope.cns.fr/microscope/transcriptomic/index.php>).

### 3. Results and discussion

#### 3.1. Genomic description of the new species MED1, named *Alteromonas plasticoclasticus*.

The coupled Illumina (short reads to obtain a quality consensus generated after assembly) and Nanopore (the long reads providing a scaffold on which the short reads are anchored) sequencing technologies revealed that the complete genome of the *MEDI* strain was composed of a unique circular chromosome of 5,332,999 bp with a GC content of 48.8%. All the general features of the sequencing approach was summarized in Supplemental materials (Fig. S1 and Table S2). The four copies of the 16S rRNA genes in the genome were not completely identical, thus re-sequencing of the complete 16S rDNA was performed using Sanger technology. The resulting sequence (identical to the ALSP\_rRNA6 and ALSP\_rRNA10 copies) was used to build a phylogenetic tree (Fig. 1). The strain appeared to be closely related to *Aestuariibacter halophilus* S23, a halophilic and aerobic strain belonging to the Alteromonadaceae family isolated from sediments originating in Korea (Yi et al., 2004). A previous study also found other isolated strains able to biodegrade PHBH films (Poly(3-Hydroxybutyrate-co-3-Hydroxyhexanoate) to be close to *A. halophilus* S23 (Moroshi et al., 2018). The Genome Taxonomy Database toolkit (GTDB-Tk) was also used for further taxonomic affiliation (Chaumeil et al. 2020). The best suggested affiliation is Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Alteromonadaceae; Alteromonas\_A. Because both phylogenetic tree and GTDB approaches assigned the isolated strain to the genus *Alteromonas* sp. but with no species affiliation, we propose the name *Alteromonas plasticoclasticus* for the *MEDI* strain.



**Figure 1:** 16S rRNA phylogenetic tree comparing different taxa close to *Alteromonas plasticoclasticus* (*MEDI* strain). The phylogenetic tree was built by the neighbor-joining without distance corrections method using the Clustal Omega software (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) and the tree data was visualized using Interactive tree of life (ItoI).

### 3.2. *Alteromonas plasticoclasticus* is a new PHBV-degrader

The discovery of this new strain was possible thanks to an original three-step protocol based on a selection of a natural biofilm growing on PHBV films after one month incubation in natural seawater, followed by the transfer of the biofilm to a MM with PHBV as carbon source for two months and the isolation of colonies by keeping the selection pressure with PHBV as carbon source (Cheng et al. 2022). To date, most of the bacterial strains identified as PHBV-degraders were tested from culture collections (Jacquin et al. 2019) and only one study isolated a novel poly(3-hydroxybutyrate) (P3HB) degrading bacteria (*Nocardioides* sp.) from plastisphere formed on plastic bag debris collected from a beach in Japan (Suzuki et al. 2021). Here, *Alteromonas plasticoclasticus* not only formed a degradation halo around the colony on the molten PHBV-containing plate but also degraded the PHBV films, as it has been confirmed by several complementary techniques used in this study.

First, we observed a drastic decrease of dissolved oxygen (average 22.5  $\mu\text{mol}$  per day) when *A. plasticoclasticus* was incubated in MM with PHBV as carbon source. Standard test procedures for estimating polymer biodegradability are commonly based on measurements of biological  $\text{O}_2$  demand or  $\text{CO}_2$  consumption, which are giving an indication on the complete biomineralization of the material by microorganisms (Harrison et al. 2018). Here, oxygen consumption was so high that the tubes were flushed 3 times with sterile air during the course of the experiment in order to avoid anoxia. Biotic and abiotic controls showed almost no oxygen consumption (0.2  $\mu\text{mol}$  per day) (Fig. 2).

Second, we used another proxy of bacterial activity based on  $^3\text{H}$ -Leucine incorporation into proteins, which is classically used in marine microbial ecology to describe heterotrophic bacterial activity (Ortega-Retuerta et al. 2012, Severin et al. 2016). We observed the highest activity in the first 11 days with a maximum of  $17.2 \pm 0.2 \text{ mgC} \cdot \text{dm}^{-2} \cdot \text{h}^{-1}$  that decreased until the end of the 40 days experiment to reach  $2.9 \pm 0.5 \text{ mgC} \cdot \text{dm}^{-2} \cdot \text{h}^{-1}$  (Fig. 2). As expected, this activity was much higher than previously observed by complex bacterial communities living on PHBV films in natural conditions (maximum of  $1.3 \cdot 10^{-5} \text{ mgC} \cdot \text{dm}^{-2} \cdot \text{h}^{-1}$ ) (Odobel et al. 2021). Abiotic controls showed no heterotrophic bacteria activity, whereas the activity of *A. plasticoclasticus* decreased when incubated in MM alone without PHBV (from  $3.7 \pm 0.2$  to  $0.1 \pm 0.03 \text{ mgC} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$  at day 0 and 40, respectively).

Another indication of PHBV degradation by *A. plasticoclasticus* was given by the measure of the weight loss, that represented 14.6% of the initial mass (loss of  $9.2 \pm 2.0 \text{ mg}$  after 40 days, eq.  $\text{xxx} \mu\text{g}/\text{cm}^2/\text{day}$ ). This value was in accordance with the literature.... We are aware that a weight loss of a polymer specimen can indicate both complete mineralization, or the formation of water-soluble degradation products only that may not be associated to complete mineralization (Haider et al. 2019). Another indirect proof of biodegradation was obtained by scanning electron microscopy (SEM) that

revealed the presence of a dense biofilm covering the entire surface of the PHBV when cultivated with no other carbon source, together with the presence of extracellular polymeric substance (EPS) (Fig. 2). It also revealed cracks on the PHBV films and a modified surface structure that are typical of bacterial degradation of the amorphous part and the apparition of a more crystalline structure called spherulite spherulitic structure went through observable morphological alteration, with clear evidences of swelling and erosion. The abiotic control with PHBV only did no showed any sign of degradation after 40 days in MM and compared to the initial PHBV film.

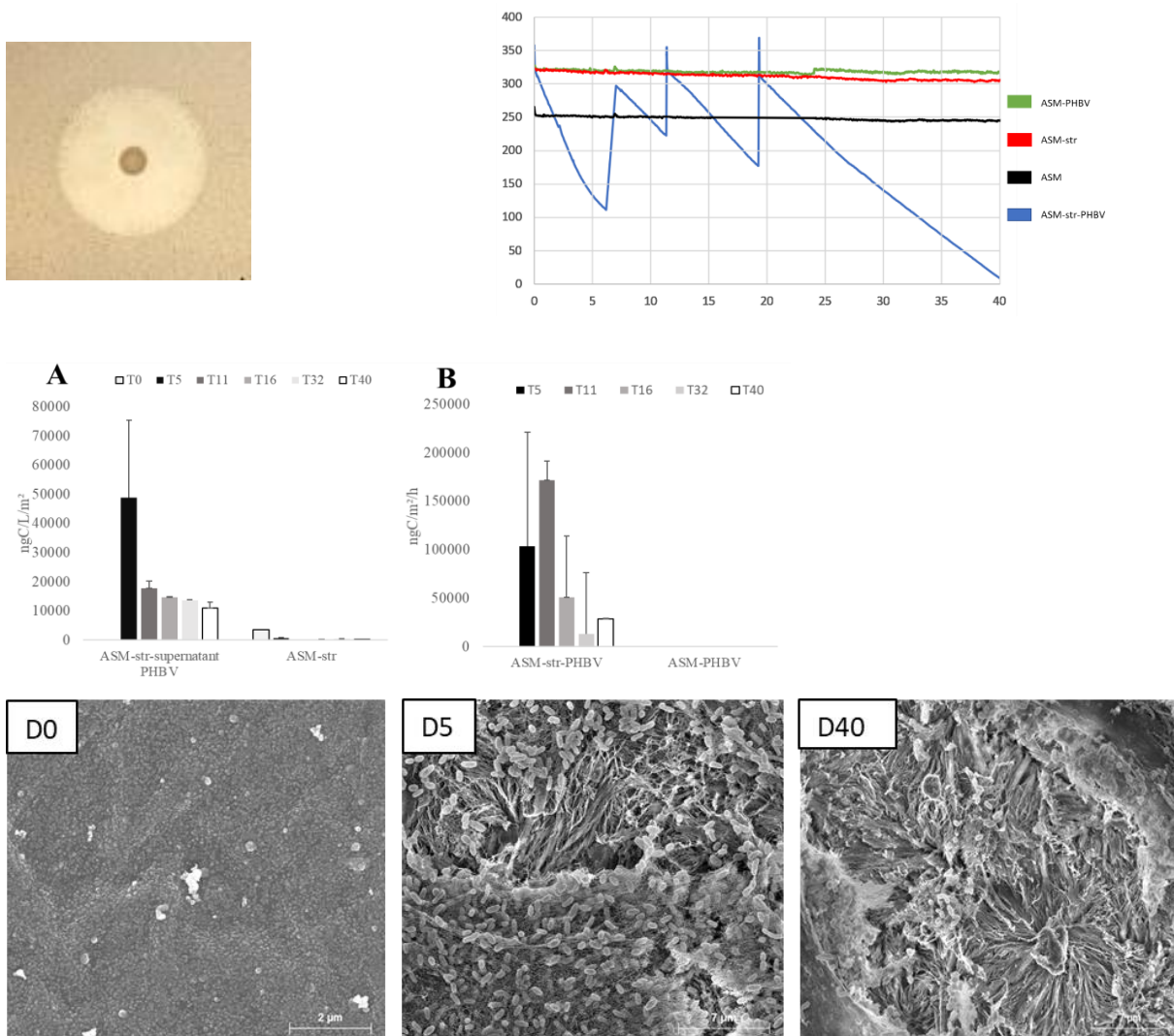


Figure 2b: Concentration of dissolved oxygen during incubation expressed as  $\mu\text{mol}$  oxygen dissolved /l per day. ASM-PHBV and ASM are the negative controls.

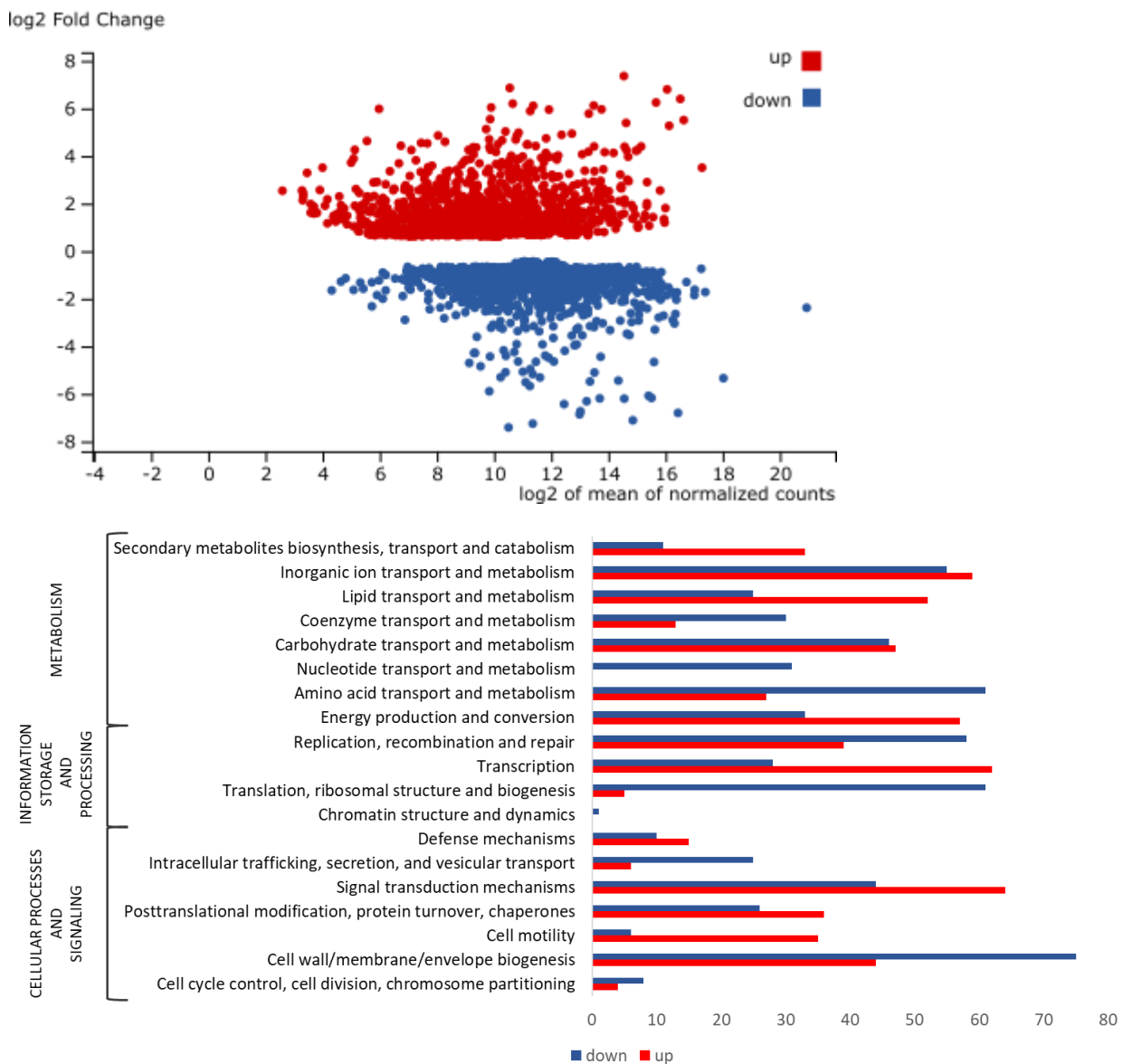
Figure 2c: Bacterial production of the strain during the incubation in artificial seawater medium (ASM) for : **A**) the negative control ASM-str and the supernatant of sample ASM-str-PHBV

expressed in ng C/l/h. **B**) the sample ASM-str-PHBV and negative control ASM-PHBV expressed in ng C /m<sup>2</sup>/h. The sampling points were the start of incubation D0, after 5 days (D5), after 11 days (D11), after 16 days (D16), after 32 days (D32) and after 40 days (D40).

Figure 2d : Scanning microscopy images of PHBV at the start of the experiment (D0) and after 5 days (D5) or 40 days (D40) of culture with the strain in the artificial medium. Spherulitis are observed on the PHBV after 40 days of incubation.

### 3.3. Characterization of the PHBV degradation pathway in *A. plasticoclasticus*

Transcriptomic analysis were performed in triplicate after 11 days of incubation in MM with PHBV or after 31 hours in MM with Glucose, i.e. corresponding to the highest bacterial activities (O<sub>2</sub> consumption, heterotrophic activity) measured during the course of the experiment (Fig. 2). Differential expression analysis showed a total of 26.4 M° to 33.6 M° reads for each triplicate under Glucose condition and 25.5 M° to 39.5 M° under PHBV condition (Table S3). A total of 1873 significant differentially expressed genes (DEG) were identified (padj ≤ 0.05, abs(log<sub>2</sub>FC>0.5)) using eggNOG (ref). We found 967 over-expressed and 906 under-expressed DEG (Fig3a) that were classified into 19 functional categories (Fig3b, Table S4). Several pathways of metabolism (“secondary metabolites biosynthesis, transport and catabolism”, “Lipid transport and metabolism” and “Energy production and conversion” and “Transcription”) were particularly overexpressed in the condition of PHBV as sole carbon source (Fig 2b, Table S4). These results confirmed previous assumption of the involvement of the beta-oxidation pathway for the *scI*-PHA assimilation (Castrejon-Godinez et al. 2019, Jacquin et al, 2019). The other up-regulated genes involved in “Cell motility” is consistent with the active microbial biofilm growing on PHA (Odobel et al. 2021, Conan et al. 2023). Clear under-expression of genes involved in “nucleotide and amino acid transport/metabolism” and “information storage and processing” gave explanation why biomass production was significantly lower when *A. plasticoclasticus* was cultivated with PHBV compared to Glucose condition.



**Figure 3:**

We then focused on the different steps involved in the PHBV degradation in order to build a general metabolic pathways by *A. plasticoclasticus* (Fig. 4). A gene cluster (ALSP\_2200 to ALSP\_2205) was identified on the complete genome of *A. plasticoclasticus* coding for permease, hydrolase, polyhydroxybutyrate depolymerase, 3-Hydroxybutyrate (3HB) dehydrogenase, NAD kinase, and carbohydrate-selective porin. In particular, two genes (ALSP\_2657 and ALSP\_2202) were identified as external depolymerases (ePHAZ) with an affinity for the short chain (scl-) PHA with an e-value of 0.0, e-110 and 4e-34 (corresponding to an identity percentage of 62%, 54% and 34%), respectively. Transcriptomic analysis showed that ALSP\_2657 gene was almost 30 times ( $\log_2FC = 4.89$ ) more expressed in PHBV condition than in Glucose condition, indicating that this enzyme ensure the first step of the biodegradation (Table 1; fig4(or5)). Then, the polymer is further



degraded via the PHB depolymerase (ALSP\_2202), coupled to hydrolase (ALSP\_2201), following the model of *Ralstonia pickettii* T1 proposed by Sugiyama et al. (2003). The hydrolysis process being not very specific (Meereboer, Misra, et Mohanty 2020), poly(R-3-hydroxybutyrate) (PHB) oligomers of various sizes and monomers of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) could be generated. These last break down complex polymers yielding smaller molecules of short chains (oligomers, dimers and monomers) are then smaller enough (< 600 Da (Jap et Walian 1996)) to pass through the bacterial membrane via the permeases (ALSP\_2200 and ALSP\_2205) and to be utilized as carbon and energy source (Shah et al. 2008).

Table 1:

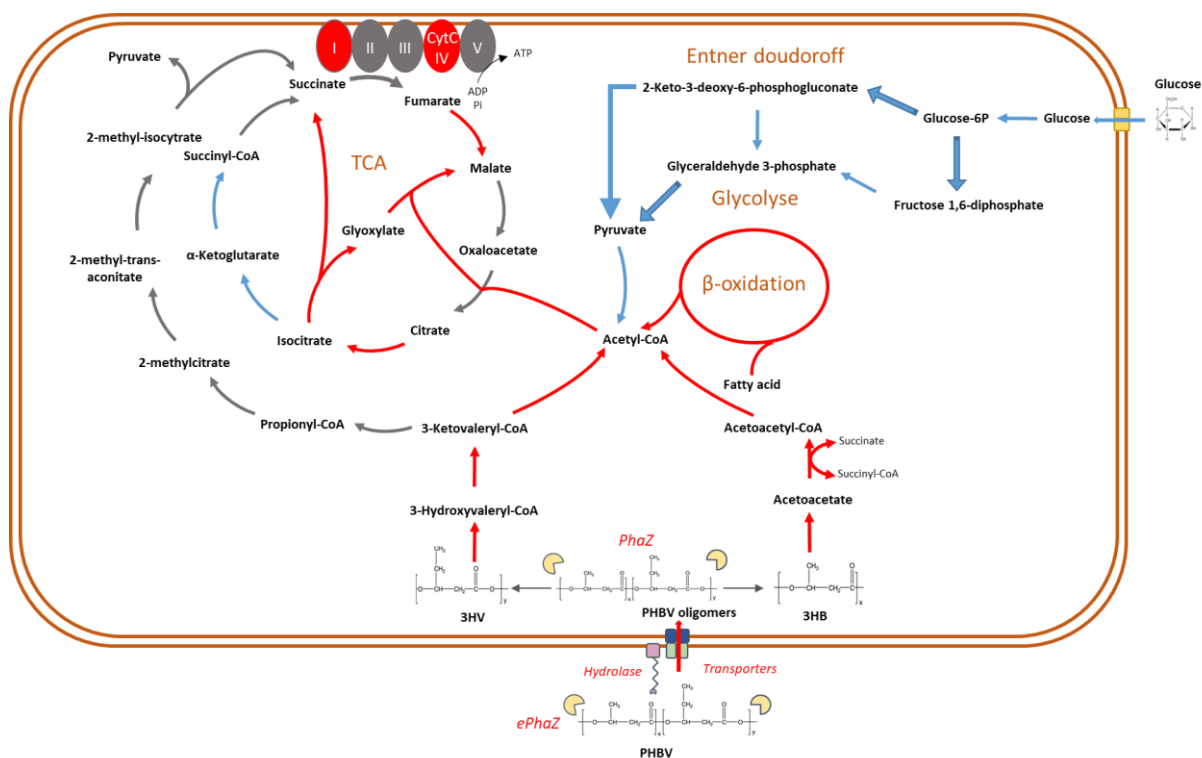
Pathway	Label	Function	Log2FC
Glycolyse	ALSP_0144	Phosphoglycerate kinase	-1.1
Glycolyse	ALSP_0247	Pyruvate dehydrogenase E1 component	-3.04
Glycolyse	ALSP_0248	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase comp	-2.89
TCA/Glycolyse	ALSP_0249	Dihydrolipoyl dehydrogenase	-1.53
Glycolyse	ALSP_1105	Pyruvate kinase II	-2.92
Entneroudoroff	ALSP_1108	Glucose-6-phosphate 1-dehydrogenase	-2.72
Entneroudoroff	ALSP_1109	6-phosphogluconolactonase, eukaryotic type	-1.95
Entneroudoroff	ALSP_1110	Phosphogluconate dehydratase	-2.16
Glycolyse	ALSP_1111	Glucokinase	-2.28
Entneroudoroff	ALSP_1112	4-hydroxy-2-oxoglutarate aldolase / 2-dehydro-3-deoxy-phosphogluconate aldolase	-2.5
Glycolyse	ALSP_1114	Glyceraldehyde-3-phosphate dehydrogenase	-2.79
TCA	ALSP_1201	2-oxoglutarate dehydrogenase E1 component	
TCA	ALSP_1202	2-oxoglutarate dehydrogenase E2 component	-1.35
Glycolyse	ALSP_2486	Enolase	-1.29
Glucose transporter	ALSP_3124	Sodium/glucose cotransporter	-1.64
Glycolyse	ALSP_3402	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	-1.04
Glucose transporter	ALSP_4807	Porin	-6.31
methylcitrate cycle	ALSP_0055	2-methyl-isocitrate dehydratase	
Beta oxidation	ALSP_0335	Acyl-coenzyme A dehydrogenase	2.4
Beta oxidation	ALSP_0370	Acyl-coenzyme A dehydrogenase	4.4
Glyoxylate Shunt	ALSP_0423	Malate synthase G	4.23
Glyoxylate Shunt	ALSP_0425	Isocitrate lyase	5.51
Beta oxidation	ALSP_0734	acyl-CoA synthetase (long-chain-fatty-acid--CoA ligase)	1.3
Beta oxidation	ALSP_0859	Long-chain fatty acid--CoA ligase	4.74
Hydroxyvalerate assimila	ALSP_0920	Acyl-CoA synthetase	3.21
Complex I Respiratory Ch	ALSP_1017	NADH dehydrogenase	1.54
Beta oxidation	ALSP_1225	Acyl-CoA dehydrogenase	4.26
Hydroxybutyrate assimila	ALSP_1251	Succinyl-CoA:3-ketoacid coenzyme A transferase subunit B	6.25
Hydroxybutyrate assimila	ALSP_1252	putative succinyl-CoA:3-ketoacid coenzyme A transferase subunit A	6.8
Hydroxyvalerate assimila	ALSP_1290	Branched-chain alpha-keto acid dehydrogenase, E1 component, alpha subunit	4.06
Hydroxyvalerate assimila	ALSP_1291	2-oxoisovalerate dehydrogenase subunit beta	4.37
Hydroxyvalerate assimila	ALSP_1292	Dihydrolipoamide acyltransferase component of branched-chain alpha-keto acid dehydroge	3.69
Respiratory chain	ALSP_1463	Cytochrome c family protein	4.49
Beta oxidation	ALSP_1559	Acyl-coenzyme A dehydrogenase	2.1
Beta oxidation	ALSP_1607	Enoyl-CoA hydratase	
Beta oxidation	ALSP_1731	Acyl-CoA dehydrogenase	3.34
Beta oxidation/Hydroxyv	ALSP_2036	3-ketoacyl-CoA thiolase	2.04
PHBV depolymerization	ALSP_2200	putative permease	4.94
PHBV depolymerization	ALSP_2201	putative hydrolase; putative acyltransferase	3.12
PHBV depolymerization	ALSP_2202	poly (3-hydroxybutyrate) depolymerase	2.01
PHBV depolymerization	ALSP_2203	3-hydroxybutyrate dehydrogenase	1.58
PHBV depolymerization	ALSP_2205	Porin	
Beta oxidation	ALSP_2035	Enoyl-CoA hydratase/3-hydroxybutyryl-CoA epimerase / 3-hydroxyacyl-CoA dehydrogenase	
Beta oxidation	ALSP_2117	Acyl-CoA synthetase	
Glyoxylate Shunt	ALSP_2577	Isocitrate dehydrogenase kinase/phosphatase	5.51
methylcitrate cycle	ALSP_2595	2-methyl-isocitrate lyase	
methylcitrate cycle	ALSP_2596	2-Methylcitrate synthase	
methylcitrate cycle	ALSP_2597	2-methylcitrate dehydratase	
methylcitrate cycle	ALSP_2598	2-methyl- aconitate isomerase	
PHBV depolymerisation	ALSP_2657	Extracellular Poly(3-hydroxybutyrate) depolymerase	4.89
TCA	ALSP_2670	Fumarate hydratase class II 2	1.33
TCA	ALSP_2976	Aconitate hydratase A	2.15
Respiratory chain	ALSP_3970	Cytochrome c oxidase polypeptide II	6.11
Respiratory chain	ALSP_3971	Cytochrome c oxidase subunit 1	5.95
Respiratory chain	ALSP_3972	Cytochrome oxidase biogenesis protein Cox11-CtaG, copper delivery to Cox1	6.04
Respiratory chain	ALSP_3973	Cytochrome c oxidase polypeptide III	4.98
Respiratory chain	ALSP_3975	SURF1-like protein	3.85
Respiratory chain	ALSP_3976	Hypotehtical protein in Cytochrome oxidase biogenesis cluster	3.19
Respiratory chain	ALSP_3977	Heme A synthase, cytochrome oxidase biogenesis protein Cox15-CtaA	2.29
Respiratory chain	ALSP_3978	Protoheme IX farnesyltransferase 2	1.99
Beta oxidation	ALSP_4483	Acetyl-CoA C-acyltransferase	
Beta oxidation	ALSP_4484	fused 3-hydroxybutyryl-CoA epimerase and delta(3)-cis-delta(2)-trans-enoyl-CoA isomeras	1.13
Beta oxidation	ALSP_4530	Acyl-CoA synthetase	
Beta oxidation/HydroxyB	ALSP_4534	putative Acetyl-CoA acetyltransferase	2.2
Beta oxidation	ALSP_4535	putative Enoyl-CoA hydratase	1.05
Beta oxidation	ALSP_4537	Acetoacetyl-CoA dehydrogenase	
Beta oxidation	ALSP_4538	Acyl-CoA dehydrogenase	
Beta oxidation	ALSP_4540	Acyl-CoA dehydrogenase	
Beta oxidation	ALSP_4545	Enoyl-CoA hydratase	1.61
Beta oxidation	ALSP_4576	Enoyl-CoA hydratase	1.21
Beta oxidation	ALSP_4606	Acyl-CoA dehydrogenase	1.53
Beta oxidation	ALSP_4607	Enoyl-CoA hydratase	0.98

Although most of the enzymes involved in  $\beta$ -oxidation cycle were overexpressed under PHBV compared to control condition (log<sub>2</sub>FC from 0.98 to 4.74, Table 1), the monomers released seemed to take the metabolic pathway previously described in *Azotobacter beijerinckii* (Senior et Dawes 1973). Indeed, 3HB monomers would be oxidized in acetoacetate by 3HB dehydrogenase using NAD<sup>+</sup> as co-factor (Sup Fig 2). Two genes coding for this enzyme (ALSP\_1227 and ALSP\_2203) were present in the genome of *A. plasticoclasticus* and both genes were slightly overexpressed in PHBV condition. Acetoacetate would be linked to a coenzyme-A by Succinyl-CoA transferase which was coded by the two co-localized and very high overexpressed sequences ALSP\_1251 and ALSP\_1252 under PHBV condition. The product acetoacetyl-CoA would be therefore cleaved into two molecules of acetyl-CoA by Acetyl-CoA acetyltransferase, an enzyme coded by several candidates which were over-expressed under PHBV condition (Table 1)(ref). The 3HV monomers would be assimilated by a bond with a coenzyme-A through the action of an Acyl-CoA synthetase. Although this enzyme is nonspecific, one overexpressed candidate under PHBV condition was identified (ALSP\_0920). The 3-hydroxyvaleryl-CoA produced would be then transformed in 3-ketovaleryl-CoA by Alpha-ketoacid dehydrogenase, a complex composed by proteins coded by three co-localized genes (ALSP\_1290, ALSP\_1291 and ALSP\_1292). Next, the 3-ketovareryl-CoA would be cleaved in acetyl-CoA and propionyl-CoA by Acetyl-CoA C-acyltransferase by 2 candidate genes (ALSP\_2036 and ALSP\_4534). Propionyl-CoA would be then metabolized by methylcitrate cycle, a pathway parallel to the tricarboxylic acid (TCA) cycle which leads to the production of succinate and pyruvate from propionyl-CoA and oxaloacetate (Brock et al. 2002). This five-steps way involves four enzymes (2-Methylcitrate synthase, 2-Methylcitrate dehydratase, 2-Methyl-aconitate isomerase and 2-Methyl-isocitrate lyase) coded by the group of co-localized genes (ALSP\_2595 - ALSP\_2598). The expression of these sequences was similar under PHBV as compared to Glucose conditions, which can be explained by the low quantity of HV in the PHBV composition.

Interestingly, the two enzymes of the glyoxylate shunt (GS), namely Isocitrate lyase (ALSP\_0425) and Malate synthase (ALSP\_0423) were both highly overexpressed under PHBV condition. Likewise, the regulatory enzyme Isocitrate dehydrogenase kinase/phosphatase coded by ALSP\_2577 was also overexpressed under PHBV condition, which inhibited the TCA enzyme Isocitrate dehydrogenase by phosphorylation to promote Isocitrate lyase and the GS (Laporte, Stueland, et Ikeda 1989). Thus, acetyl-CoA was more involved in supplying the GS than the TCA cycle. The preferential use of the GS rather than complete TCA cycle can be explained by the fact that this bypass prevents the loss of the carbons as CO<sub>2</sub>, avoiding the TCA cycle decarboxylation steps (Dolan et Welch 2018), and the expense of energy production (de la Peña Mattozzi, Kang, et Keasling 2010). Moreover, the GS is essential when bacteria are cultured with carbon sources that

enter metabolism exclusively as acetyl-CoA (Laporte, Stueland, et Ikeda 1989), which is the case for PHBV. However, the GS bypasses the electron carriers production steps of the TCA cycle, in particular NADH production, which are required within the electron transport chain in the production of ATP (Koedooder et al. 2018). Nevertheless, this phenomenon can be compensated by enhanced NADH and FADH<sub>2</sub> production through stimulation of the  $\beta$ -oxidation cycle under PHBV condition. The reduced electron carriers were oxidized by the membrane respiratory chain complexes. The expression of the first three complexes did not change between the two culture conditions. In contrast, Cytochrome c (ALSP\_1463) and complex IV corresponding to Cytochrome c oxidase (composed of several subunits which are coded by a group of co-localized genes (ALSP\_3970 - ALSP\_3978) were overexpressed in PHBV condition. It is commonly accepted that prokaryotes are able to respond to environmental changes by inducing particular respiratory components upon demand (Ludwig 1987). Overexpression of Cytochrome c oxidase stimulated the pumping of protons from cytoplasm to periplasm and thus generates a high electrochemical gradient in *A. plasticoclasticus*. This enzyme was used for the functioning of ATP synthase, formed by many subunits coded by the group of co-localized genes (ALSP\_3816 - ALSP\_3824). Their expression was similar under PHBV and Glucose conditions, indicating that all metabolic pathways set up upstream by *Alteromonas plasticoclasticus* were sufficient to ensure ATP synthase function and adequate cell energy production.

Finally, we observed that most of the enzymes involved in the glycolysis and Entner doudoroff pathways were underexpressed under PHBV condition (Table 1) compared to the control condition with glucose, as well as the specific transporter of this monosaccharide (ALSP\_4807). This result seems logical since bacteria will not spend energy to translate and transcribe proteins that are not useful in the culture conditions in which they grow.



**Figure 4:** Overview of the PHBV (red arrows) and Glucose (blue arrows) degradation pathways used by *Alteromonas plasticoclasticus*, as revealed by genomic and transcriptomic analysis. Abbreviation : TCA, tricarboxylic acid cycle, ePHAZ, extracellular depolymerase catalyzing the degradation of exogenous PHA, iPHAZ, intracellular depolymerase catalyzing the degradation of endogenous PHA.

#### 4. Conclusion

This is the first report on the genus *Alteromonas* exhibiting ability to degrade PHBV, which was isolated from the marine environment plastisphere. The strain is able to form biofilm on the film surface and perform a complete mineralization of PHBV as sole carbon source. We identified two external depolymerase (ePHAZ) with its catalytic triad catalytic triad (Ser-His-Asp) that degrade PHBV into 3HB oligomers and monomers that enter the cells for further mineralization through the glyoxylate shunt. Previous studies underlined the potential of the plastisphere as a new genetic reservoir for discovering new processes involved in plastic production and biodegradation (Jacquin et al. 2019, Suzuki et al. 2021). In particular, some PHA-degrading bacteria may also present PHA production capabilities (Ma et al., 2011; Ray and Kalia, 2017) and further studies are needed to evaluate this potential by *Alteromonas plasticoclasticus*. We believe that this study open new routes for a better understanding of plastic biodegradation in the marine environment.

## References

- Abràmoff M, Magalhães P, and Ram S. 2004. Image processing with ImageJ. *biophotonics international* **11**: 36–42.
- Ali I and Jamil N. 2016. Polyhydroxyalkanoates: Current applications in the medical field. *Front Biol* **11**: 19–27.
- Amaral-Zettler LA, Zettler ER, and Mincer TJ. 2020. Ecology of the plastisphere. *Nat Rev Microbiol* **18**: 139–51.
- Brock M, Maerker C, Schütz A, Völker U, Buckel W. Oxidation of propionate to pyruvate in *Escherichia coli*. Involvement of methylcitrate dehydratase and aconitase. *Eur J Biochem*. 2002 Dec;269(24):6184-94. doi: 10.1046/j.1432-1033.2002.03336.x.
- Chang H-F, Chang W-C, and Tsai C-Y. 2012. Synthesis of poly(3-hydroxybutyrate/3-hydroxyvalerate) from propionate-fed activated sludge under various carbon sources. *Bioresour Technol* **113**: 51–7.
- Donlan RM. 2002. Biofilms: microbial life on surfaces. *Emerging Infect Dis* **8**: 881–90.
- Dussud C, Hudec C, George M, *et al.* 2018. Colonization of Non-biodegradable and Biodegradable Plastics by Marine Microorganisms. *Front Microbiol* **9**.
- Eubeler JP, Zok S, Bernhard M, and Knepper TP. 2009. Environmental biodegradation of synthetic polymers I. Test methodologies and procedures. *TrAC Trends in Analytical Chemistry* **28**: 1057–72.
- Fegatella F, Lim J, Kjelleberg S, and Cavicchioli R. 1998. Implications of rRNA operon copy number and ribosome content in the marine oligotrophic ultramicrobacterium *Sphingomonas* sp. strain RB2256. *Appl Environ Microbiol* **64**: 4433–8.
- Gorke JT, Okrasa K, Louwagie A, *et al.* 2007. Enzymatic synthesis of poly(hydroxyalkanoates) in ionic liquids. *Journal of Biotechnology* **132**: 306–13.
- Grimaud R, Ghiglione J-F, Cagnon C, *et al.* 2012. Genome sequence of the marine bacterium *Marinobacter hydrocarbonoclasticus* SP17, which forms biofilms on hydrophobic organic compounds. *Journal of Bacteriology* **194**: 3539–40.
- Handrick R, Reinhardt S, and Jendrossek D. 2000. Mobilization of poly(3-hydroxybutyrate) in *Ralstonia eutropha*. *J Bacteriol* **182**: 5916–8.
- Handrick R, Reinhardt S, Kimmig P, and Jendrossek D. 2004. The “intracellular” poly(3-hydroxybutyrate) (PHB) depolymerase of *Rhodospirillum rubrum* is a periplasm-located protein with specificity for native PHB and with structural similarity to extracellular PHB depolymerases. *J Bacteriol* **186**: 7243–53.

- Jacquín J, Callac N, Cheng J, Giraud C, Pujo-Pay M, Conan P, Meistertzheim A-L, Barbe V, Bruzard S, Ghiglione JF. 2021. Microbial diversity and activity during the biodegradation in seawater of various substitutes to conventional plastic cotton swabs sticks. *Frontiers in Microbiology*. Jul 15;**12**: 1462.
- Jacquín J, Cheng J, Odobel C, Pandin C, Conan P, Pujo-Pay M, Barbe V, Meistertzheim AL, Ghiglione JF. 2019. Microbial Ecotoxicology of Marine Plastic Debris: A Review on Colonization and Biodegradation by the “Plastisphere.” *Front Microbiol* Apr 25;**10**:865.
- Jambeck JR, Geyer R, Wilcox C, *et al.* 2015. Marine pollution. Plastic waste inputs from land into the ocean. *Science* **347**: 768–71.
- Jendrossek D. 2005. Extracellular Polyhydroxyalkanoate (PHA) Depolymerases: The Key Enzymes of PHA Degradation. In: *Biopolymers Online*. American Cancer Society.
- Jendrossek D. 2007. Peculiarities of PHA granules preparation and PHA depolymerase activity determination. *Appl Microbiol Biotechnol* **74**: 1186–96.
- Jendrossek D and Handrick R. 2002. Microbial degradation of polyhydroxyalkanoates. *Annu Rev Microbiol* **56**: 403–32.
- Knoll M, Hamm TM, Wagner F, *et al.* 2009. The PHA Depolymerase Engineering Database: A systematic analysis tool for the diverse family of polyhydroxyalkanoate (PHA) depolymerases. *BMC Bioinformatics* **10**: 89.
- Kong D, Li W, Deng Y, *et al.* 2018. Denitrification-Potential Evaluation and Nitrate-Removal-Pathway Analysis of Aerobic Denitrifier Strain *Marinobacter hydrocarbonoclasticus* RAD-2. *Water* **10**: 1298.
- Kumar S, Nei M, Dudley J, and Tamura K. 2008. MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform* **9**: 299–306.
- Lebaron P, Ghiglione J-F, Fajon C, *et al.* 1998. Phenotypic and genetic diversity within a colony morphotype. *FEMS Microbiol Lett* **160**: 137–43.
- Liu Z, Zhu Z, Yang J, *et al.* 2019. Domain-centric dissection and classification of prokaryotic poly(3-hydroxyalkanoate) synthases. *bioRxiv*: 693432.
- Maestro B and Sanz JM. 2017. Polyhydroxyalkanoate-associated phasins as phylogenetically heterogeneous, multipurpose proteins. *Microbial Biotechnology* **10**: 1323–37.
- Martinez Tobon DI. 2019. Biodegradation of Polyhydroxybutyrate by Bacterial Strains, Native Extracellular PHB Depolymerases, and Structural Variants.
- Meng D-C, Shen R, Yao H, *et al.* 2014. Engineering the diversity of polyesters. *Curr Opin Biotechnol* **29**: 24–33.

- Mezzolla V, D'Urso OF, and Poltronieri P. 2018. Role of PhaC Type I and Type II Enzymes during PHA Biosynthesis. *Polymers (Basel)* **10**.
- Mothes G, Ackermann J-U, and Babel W. 2004. Mole Fraction Control of Poly([R]-3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB/HV) Synthesized by *Paracoccus denitrificans*. *Engineering in Life Sciences* **4**: 247–51.
- Oberbeckmann S, Löder MGJ, and Labrenz M. 2015. Marine microplastic-associated biofilms – a review. *Environ Chem* **12**: 551–62.
- Ong SY, Chee JY, School of Biological Sciences Universiti Sains Malaysia, *et al.* 2017. Degradation of Polyhydroxyalkanoate (PHA): a Review. *J Sib Fed Univ, Biol* **10**: 21–225.
- Peña C, López S, García A, *et al.* 2014. Biosynthesis of poly- $\beta$ -hydroxybutyrate (PHB) with a high molecular mass by a mutant strain of *Azotobacter vinelandii* (OPN). *Ann Microbiol* **64**: 39–47.
- Reis MAM, Serafim LS, Lemos PC, *et al.* 2003. Production of polyhydroxyalkanoates by mixed microbial cultures. *Bioprocess Biosyst Eng* **25**: 377–85.
- Simon M and Azam F. 1989. Protein content and protein synthesis rates of planktonic marine bacteria. *Marine Ecology Progress Series* **51**: 201–13.
- Sudesh K, Abe H, and Doi Y. 2000. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Progress in Polymer Science* **25**: 1503–55.
- Tebaldi ML, Maia ALC, Poletto F, *et al.* 2019. Poly(-3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV): Current advances in synthesis methodologies, antitumor applications and biocompatibility. *Journal of Drug Delivery Science and Technology* **51**: 115–26.
- Tsuyoshi N and Tetsuya Y. 2006. Acetyl-CoA acyltransferase gene disrupted bacterium for producing polyhydroxyalkanoate and method for producing polyhydroxyalkanoate using the same.
- Verlinden R a. J, Hill DJ, Kenward MA, *et al.* 2007. Bacterial synthesis of biodegradable polyhydroxyalkanoates. *J Appl Microbiol* **102**: 1437–49.
- Wambeke FV, Ghiglione J-F, Nedoma J, *et al.* 2009. Bottom up effects on bacterioplankton growth and composition during summer-autumn transition in the open NW Mediterranean Sea. *Biogeosciences* **6**: 705–20.
- Wang Y, Chen R, Cai J, *et al.* 2013. Biosynthesis and Thermal Properties of PHBV Produced from Levulinic Acid by *Ralstonia eutropha*. *PLOS ONE* **8**: e60318.

Vallenet, David, Alexandra Calteau, Mathieu Dubois, Paul Amours, Adelme Bazin, Mylène



Beuvin, Laura Burlot, et al. 2020. “MicroScope: An Integrated Platform for the Annotation and Exploration of Microbial Gene Functions through Genomic, Pangenomic and Metabolic Comparative Analysis.” *Nucleic Acids Research* 48 (D1): D579–89.

Li H. (2013). Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *ArXiv*

Li H., Handsaker B., Wysoker A., Fennell T., Ruan J., Homer N., et al. (2009). The sequence alignment/Map format and SAMtools. *Bioinformatics* 25 2078–2079. 10.1093/bioinformatics/btp352

Quinlan, Aaron R., and Ira M. Hall. 2010. “BEDTools: A Flexible Suite of Utilities for Comparing Genomic Features.” *Bioinformatics* 26 (6): 841–42.

Love, Michael I., Wolfgang Huber, and Simon Anders. 2014. “Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2.” *Genome Biology* 15 (12): 550.

Klingbeil, Kroppenstedt and Jendrossek; FEMS Microbiology Letters 142 (1996) 215-221 ; Taxonomic identification of *Streptomyces exfoliatus* K10 and characterization of its poly(3-hydroxybutyrate) depolymerase gene

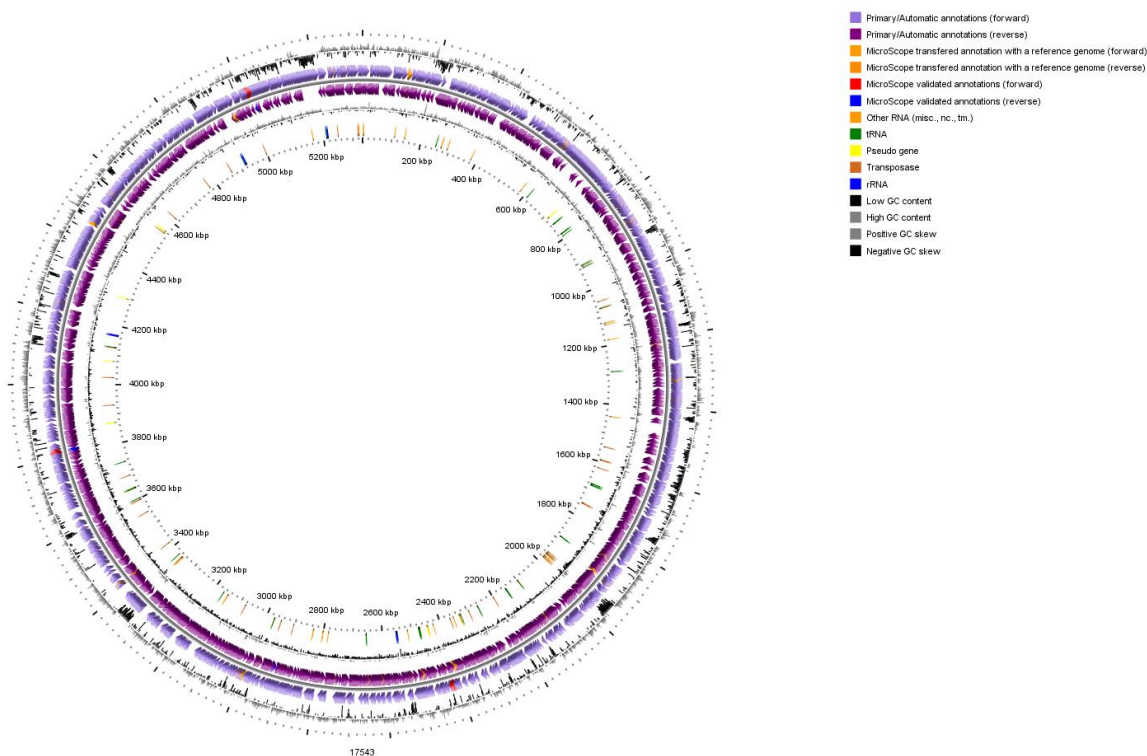


Figure S1: Circular map of *Alteromonas* sp. genome

Table S1 : Composition of the minimum medium (MM) (modified from Fegatella et al. 1998) : NaCl, 420 mM; Tris, 50 mM; Na<sub>2</sub>SO<sub>4</sub>, 28.8 mM; KCl, 9.39 mM; NaBr, 0.84 mM; H<sub>3</sub>BO<sub>3</sub>, 0.485 mM; MgCl<sub>2</sub> 6H<sub>2</sub>O, 0.546 mM; CaCl<sub>2</sub>, 10.5 mM; NH<sub>4</sub>Cl, 9.35 mM; SrCl<sub>2</sub> 6H<sub>2</sub>O, 0.0638 mM; NaF, 0.0714 mM; NaNO<sub>3</sub>, 0.88 mM; NaH<sub>2</sub>PO<sub>4</sub> H<sub>2</sub>O, 0.036 mM; KH<sub>2</sub>PO<sub>4</sub>, 0.106 mM; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.04 μM; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.08 μM; CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.04 μM; MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.9 μM; Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.03 μM; FeCl<sub>3</sub>, 1.85 μM; thiamine, 0.3 μM; biotin, 2.1 nM; and B12 vitamin, 0.317 nM (Fegatella et al., 1998).

Temporal monitoring of nutrients in the artificial seawater environment.

ASM-str PHBV	PO4	NO2	NO3+NO 2
D0	107	0.0488	1711
D52	90.6	0.0968	1727
D11	47.5	1.2828	1701
D16	43.75	0.9888	1605
D32	53.6	3.0868	1651
D40	26.8	2.1428	1659

Table S2: General features of *Alteromonas* sp. genome

Attribute	Values
Genome size (bp)	5,332,999
DNA G+C (%)	48.8
Plasmid	0
DNA scaffolds	1
rRNA genes	12
tRNA genes	58
tmRNA	1
misc_RNA	34
Protein	4810
Predicted genes	4805
Pseudo genes	8

Table S3

	Triplicate	Total read number	Nb of reliable reads	Pourcentage of reliable reads (%)
PHBV	PHBV-1	25530930	23542060	92.21
	PHBV-2	39504894	35990661	91.10
	PHBV-3	30428591	28997536	95.30
Glucose	GLC-4	26442568	17516221	66.24
	GLC-5	33609136	31979344	95.15
	GLC-6	29812037	28507823	95.63

Table S4

Process	Class ID	Description	over-expressed			Under-expressed		
			gene fraction in comparison	genes	% of genes in comparison	gene fraction in comparison	Genes	% of genes in comparison
CELLULAR PROCESSES AND SIGNALING	D	Cell cycle control, cell division, chromosome partitioning	4/841	4	0.4756 %	8/852	8	0.9390 %
	M	Cell wall/membrane/envelope biogenesis	44/841	44	5.2319 %	75/852	75	8.8028 %
	N	Cell motility	35/841	35	4.1617 %	6/852	6	0.7042 %
	O	Posttranslational modification, protein turnover, chaperones	36/841	36	4.2806 %	26/852	26	3.0516 %
	T	Signal transduction mechanisms	64/841	64	7.6100 %	44/852	44	5.1643 %
	U	Intracellular trafficking, secretion, and vesicular transport	6/841	6	0.7134 %	25/852	25	2.9343 %
	V	Defense mechanisms	15/841	15	1.7836 %	10/852	10	1.1737 %
INFORMATION STORAGE AND PROCESSING	B	Chromatin structure and dynamics				1/852	1	0.1174 %
	J	Translation, ribosomal structure and biogenesis	5/841	5	0.5945 %	61/852	61	7.1596 %
	K	Transcription	62/841	62	7.3722 %	28/852	28	3.2864 %
METABOLISM	L	Replication, recombination and repair	39/841	39	4.6373 %	58/852	58	6.8075 %
	C	Energy production and conversion	57/841	57	6.7776 %	33/852	33	3.8732 %
	E	Amino acid transport and metabolism	27/841	27	3.2105 %	61/852	61	7.1596 %
	F	Nucleotide transport and metabolism				31/852	31	3.6385 %
	G	Carbohydrate transport and metabolism	47/841	47	5.5886 %	46/852	46	5.3991 %
	H	Coenzyme transport and metabolism	13/841	13	1.5458 %	30/852	30	3.5211 %
	I	Lipid transport and metabolism	52/841	52	6.1831 %	25/852	25	2.9343 %
	P	Inorganic ion transport and metabolism	59/841	59	7.0155 %	55/852	55	6.4554 %
	Q	Secondary metabolites biosynthesis, transport and catabolism	33/841	33	3.9239 %	11/852	11	1.2911 %
POORLY CHARACTERIZED	S	Function unknown	268/841	268	31.8668 %	235/852	235	27.5822 %

**Titre :** Les polyhydroxyalcanoates (PHA) en milieu marin : production bactérienne à façon et biodégradabilité

**Mots clés :** Polyhydroxyalcanoates, production, biodégradation, environnement marin

**Résumé :** La production de matières plastiques a dépassé les 390 millions de tonnes par an à partir de 2021 et ne cesse d'augmenter. Ces matériaux aux propriétés versatiles et aux coûts de fabrication faibles se retrouvent dans tous les secteurs d'activités. Paradoxalement aux bénéfices apportés par l'introduction massive des matériaux plastiques à notre société, leur fin de vie représente un inconvénient majeur d'un point de vue environnemental. Des solutions de lutte contre la pollution plastique ont récemment été mises en place au niveau gouvernemental et sociétal dans de nombreux pays afin de limiter l'impact des plastiques sur l'environnement. Parmi ces solutions, le développement de plastiques biodégradables, se profilent depuis les

dernières décennies pour répondre à des secteurs d'activités ne pouvant se passer de plastiques. Parmi les plastiques biodégradables, les polyhydroxyalcanoates (PHA) sont des polymères d'origine bactérienne et biosourcés. Cette thèse vise à mieux comprendre leur éventuelle fin de vie dans l'environnement marin, les mécanismes de leur biodégradation ainsi que les microorganismes associés afin de mieux définir leur production et leur usage. Les connaissances apportées sur le comportement de ces polymères dans l'environnement marin en fonction des propriétés intrinsèques qu'ils peuvent présenter sont nécessaires pour adapter leur production et mieux définir leurs usages.

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**Title :** Polyhydroxyalkanoates (PHA) in marine environment : Tailor-made bacterial production and biodegradability

**Keywords :** Polyhydroxyalkanoates, production, biodegradation, marine environment

**Abstract :**

The production of plastic materials exceeded 390 million tons per year from 2021 and continues to increase. These materials with versatile properties and low manufacturing costs are found in all sectors of activity. Paradoxically to the benefits brought by the massive introduction of plastic materials in our society, their end of life represents a major disadvantage from an environmental point of view. Solutions to fight plastic pollution have recently been put in place at governmental and societal level in many countries in order to limit the impact of plastics on the environment. Among these solutions, the development of biodegradable plastics has been emerging for the past decades to respond to sectors of activity that cannot do without plastics. Among biodegradable plastics, polyhydroxyalkanoates (PHA) are polymers of

bacterial origin and biosourced. This thesis aims to better understand their possible end of life in the marine environment, the mechanisms of their biodegradation as well as the associated microorganisms in order to better define their production and use. The knowledge provided on the behavior of these polymers in the marine environment according to the intrinsic properties that they may present are necessary to adapt their production and better define their use.

