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Audrey MAGNIN

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Dégradation de polyuréthanes thermoplastiques par voies enzymatique et microbienne. Vers un recyclage biologique et durable.

THÈSE dirigée par :

M. AVEROUS Luc	Professeur, Université de Strasbourg
M. PHALIP Vincent	Professeur, Université de Lille

RAPPORTEURS :

M. PAUL Etienne	Professeur, INSA Toulouse
M. PERUCH Fréderic	Directeur de recherches CNRS, LCPO Bordeaux

---

AUTRES MEMBRES DU JURY :

Mme. BEGIN Sylvie	Professeure, Université de Strasbourg
Mme. CARAPITO Christine	Chargée de recherche CNRS, IPHC Strasbourg

ICPEES (Institut de chimie et procédés pour l'énergie, l'environnement et la santé) – UMR 7515

25 rue Becquerel – 67087 Strasbourg Cedex 2

Communications liées aux travaux de thèse

Article publié

Magnin A., Hoornaert L., Pollet E., Laurichesse S., Phalip V. and Avérous L., - “Isolation and characterization of different promising fungi for biological waste management of polyurethanes”, *Microbial Biotech*, 2018, DOI: 10.1111/1751-7915.13346

Chapitre de livre

Wierckx, N., Narancic T., Eberlein C., Wei R., Drzyzga O., Magnin A., Ballerstedt H., et al. "Plastic Biodegradation: Challenges and Opportunities." *In Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, 1-29: Springer, 2018. DOI: 10.1007/978-3-319-44535-9_23-1

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Magnin A., Pollet E., Perrin R., Ullmann C., Persillon C., Phalip V., Avérous L. - “Enzymatic Recycling of Thermoplastic Polyurethanes: Synergistic Effect of an Esterase and an Amidase and Recovery of Building Blocks”, Submitted in *Waste management*

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Magnin A., François Delalande F., Aurélie Hirschler A., Eric Pollet E., Vincent Phalip V., Carapito C., Luc Avérous L. - “Exoproteome analysis of Aspergillus oryzae and Penicillium brasilianum grown on neat polyesters and polyurethane substrates”

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Liste des abréviations

1,4-BDO	1,4-Butanediol
2,4'-TDA	2,4'-Toluene diamine
2,4-TDI	2,4'-Toluene diisocyanate/2,4'-Diisocyanate de toluène
4,4'-MDA/MDA	4,4'-Methylène dianiline
4,4'-MDI/MDI	4,4'-Methylene diisocyanate/4,4'-diisocyanate de diphénylméthylène
ASTM	American Society for Testing and Materials
BSA	Bovine Serum Albumine
ButOH	Butanol
CFU	Colony forming unit
CPM	Counts per minute
DMPA	Dimethyl propionic acid
DMSO	Dimethyl sulfoxide
Đ	Dispersity
EC number	Enzyme Commission number
EDTA	Ethylenediaminetetraacetic acid
EU/UE	European Union/Union Européenne
DFA	Fatty acid dimer/Dimère d'acide gras
FC	Fold Change
FA	Formic Acid
FTIR	Fourier-transform infrared spectroscopy/ Spectroscopie infrarouge à transformée de Fourier
ITS	Internal Transcribed Spacer
GC	Gaz Chromatography
H12MDI	Hexamethylene diisocyanate
HDI	Hexane diisocyanate
H2020	Horizon 2020
IPDI	Isophorone diisocyanate
LC	Liquide Chromatography
LDI	Lysine diisocyanate
MS	Mass Spectrométrie/Spectrométrie de masse
MALDI TOF	Matrix Assisted Laser Desorption Ionisation - Time of Flight
Mt	Millions tons
MM	Minimal medium/Milieu minimum
NCBI	National Center for Biotechnology Information
NMR/RMN	Nuclear Magnetic Resonnance/Resonnance Magnetique Nucléaire
Mn	Number average molecular weight
Mw	Weight average molecular weight

PBS	Phosphate Buffer Saline
pNAA	pNitroacetaniline
pNP	pNitrophenol
pNPA	pNitrophenol acetate
pNPB	pNitrophenol butyrate
PHEC	Poly(1,6-hexyl 1,2-ethyl carbonate)
PEG	Poly(ethylene glycol)
PHA	Poly(hydroxy alcanoate)
PPC	Poly(propylene carbonate)
PPG	Poly(propylene glycol)
PBA	Polybutylene adipate
PCL	Polycaprolactone
PEs	Polyester
PET	Polyethylene terephthalate
PLA	Polylactic acid
PTHF/PTMEG	Polytetrahydrofurane/Polytetramethyleneglycol
PUD	Polyurethane dispersion
PU	Polyurethanes/Polyuréthanes
PDA	Potatoe Dextrose Agar
PDB	Potatoe Dextrose Broth
pTSA	p-toluenesulfonamide
pTSI	p-toluenesulfonyl isocyanate
rpm	Rotation per minute
SDS	Sodium Dodecyl Sulftae
SEM	Scanning Electron Microscopy
SEC	Size exclusion chromatography/Chromatographie d'exclusion stérique
Std. Dev.	Standard Deviation
MS/MS	Tandem Mass Spectrométrie/Spéctrométrie de masse en tandem
THF	Tetrahydrofurane
TGA	Thermogravimetric analysis
TPU	Thermoplastic polyuretanes/Polyuréthanes thermoplastiques
TOC	Total Organic Carbon
TPG	Tripropylene glycol

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Introduction générale : Le recyclage biologique des matières plastiques

1. La pollution liée aux matières plastiques

Les matières plastiques font partie intégrante de notre quotidien et contribuent à notre confort de vie. Elles se déclinent sous une infinité de formes et répondent à un très grand nombre d'applications. C'est un secteur qui présente une croissance continue, en 2016 par exemple, 335 millions de tonnes (Mt) de matières plastiques ont été produites dans le monde dont 60 en Europe (PlasticsEurope 2017).

Cependant, les matières plastiques sont une source majeure de pollution de par leur production, mise en œuvre, utilisation et de par les très nombreux déchets générés. La production de matière plastique fait, en grand majorité, appel à l'utilisation de ressources fossiles. Dans une approche de développement durable et de chimie verte, la transition de ressources fossiles à la biomasse pour produire des polymères biosourcés, potentiellement biodégradables, est l'un des défis majeurs de notre époque (Hillmyer 2017).

En 2016, 27,1 millions de tonnes de déchets plastiques ont été collectés. Trois méthodes sont ensuite communément décrites pour traiter ces déchets : la mise en décharge (27,3%), la conversion en énergie (41,6%, majoritairement via incinération) et le recyclage (31,1%) (PlasticsEurope 2017). Pour la première fois, la proportion de matière plastique recyclée dépasse celle de mise en décharge démontrant ainsi la prise de conscience collective autour de cette démarche. Cependant, la quantité de déchets plastiques non-collectés ainsi que celle mise en décharge représentent toujours une source de pollution importante pour l'environnement.

Les conséquences environnementales sont déjà mesurables. Les déchets plastiques, généralement légers, sont transportés par le vent et les cours d'eau vers les océans où ils s'accumulent (Cozar et al. 2014). Cette accumulation se fait majoritairement sous la forme de micro-plastiques, de taille inférieure à 1 mm (Galloway and Lewis 2016). Ces micro-plastiques correspondent aux déchets fragmentés par des contraintes physiques et aux microbilles largement utilisées dans les produits cosmétiques. Parmi ces déchets, les polymères les plus décrits sont le polyéthylène, le polypropylène et le polystyrène (PS) (O'Connor, Golsteijn, and Hendriks 2016). Cependant, un panel de polymères bien plus large, incluant notamment les polyuréthanes (PU), les polyamides (PA) ou le poly(éthylène téréphtalate) (PET), est en réalité

retrouvé dans les océans (Frère et al. 2016; Turner and Lau 2016; Suaria et al. 2016) formant ce que l'on appelle parfois le « septième continent ».

Les déchets plastiques hydrophobes attirent à leur surface des micro-polluants organiques (pesticides, perturbateurs endocriniens…) devenant ainsi hautement toxiques. Les composés issus de polymères synthétiques entrent dans la chaîne alimentaire, en grande partie par le biais d'animaux marins. L'accumulation de ces polluants dans le tractus gastrique des animaux limite la possibilité d'ingurgiter de la nourriture. Les conséquences sur la santé tout au long de la chaîne alimentaire peuvent être dramatiques (Figure I.1). La toxicité de ces composés fait actuellement l'objet de nombreuses recherches. Les écosystèmes terrestres sont aussi concernés par cette menace à moyen terme (Rochman et al. 2013).

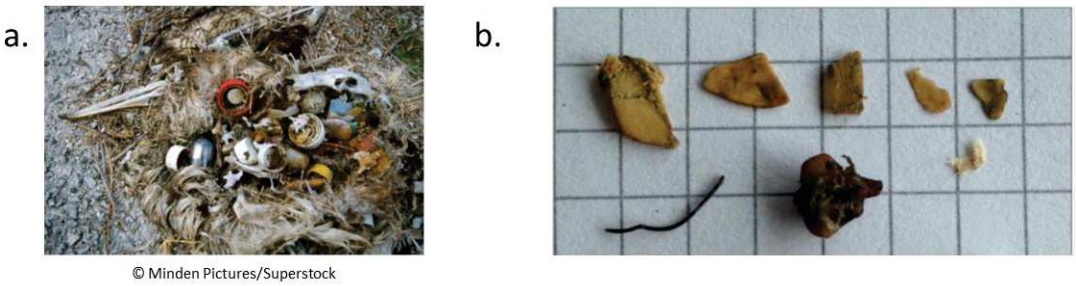

Figure I.1 – (a) Cadavre d'un albatros de Laysan (Phoebastria immutabilis) contenant des débris plastiques ingérés en mer, îles Midway, Hawaii (Gross 2013), (b) Morceaux collectés dans le tractus gastrique d'un fulmar boréal (Fulmarus glacialis) représentant une masse totale de 80 mg (grille de 1cm x 1cm) (Poon et al. 2017)

2. Le recyclage biologique des matières plastiques

Pour limiter ces nuisances environnementales, plusieurs solutions émergent. Une consommation raisonnée passant par une réglementation stricte est souhaitée. En France, les sacs plastiques à usages uniques sont interdits en caisse de supermarché depuis le 1er Juin 2016 et interdits dans tous les types de commerces depuis le 1er Janvier 2017[1]. Les sacs plastiques à usage unique ont été remplacés par des sacs réutilisables, en papier ou compostables. Le 28 mai 2018, la Commission Européenne a soumis une directive ciblant la réduction de la consommation de 10

[1] https://www.economie.gouv.fr/dgccrf/sacs-en-plastique-jetables-cest-fini

produits en plastique à usage unique[2]. Cinq d'entre eux (les bâtonnets de coton-tige, les couverts et vaisselles jetables, les pailles, les agitateurs à boissons et les bâtons utilisés pour fixer les ballons gonflables) pourraient être retirés du marché dès 2021.

Le remplacement des matières plastiques conventionnelles par des matériaux biodégradables et le développement de méthodes de valorisation efficaces en fin de vie sont des solutions pour lutter contre cette pollution croissante. Cette dernière voie permettrait, à terme, de percevoir les matières plastiques en fin de vie non comme des déchets mais comme une nouvelle source de matière première valorisable. Cela répond au principe d'économie circulaire dans une approche de développement durable (Huysman et al. 2017). Différentes voies telles que le recyclage thermique ou chimique sont actuellement développées dans divers secteurs industriels.

Plusieurs freins technologiques et économiques empêchent le déploiement de méthodes de recyclage efficaces (Ignatyev, Thielemans, and Vander Beke 2014). Le prix des polymères recyclés est souvent plus élevé que celui des matières plastiques vierges. Une taxation adaptée (écotaxe) et une augmentation du prix des ressources fossiles liée à la raréfaction de certaines d'entre elles pourraient cependant inverser cette tendance dans les décennies à venir. De plus, une perte des propriétés des polymères est généralement observée au cours des procédés de recyclage résultant d'une modification des structures et des masses molaires (Singh et al. 2017). Ainsi, le nombre de cycles est limité.

Ces dernières années ont vu l'émergence d'un nouveau type de recyclage : le recyclage biologique. Celui-ci repose sur l'utilisation de catalyseurs biologiques : les enzymes, pour dépolymériser les macromolécules constitutives des matériaux plastiques (Wei and Zimmermann 2017). Cette voie s'inscrit dans une approche respectueuse de l'environnement (chimie verte) car elle se fait à de faibles températures, généralement en dessous de 70°C, et peu d'énergie est donc consommée. De plus, aucun intermédiaire chimique, potentielle source de pollution, n'est nécessaire à ces réactions.

[2] https://ec.europa.eu/info/law/better-regulation/initiatives/com-2018-340_en

Le programme Horizon 2020 (H2020) de la Commission Européenne, démarré le 1er janvier 2014, apporte un soutien financier pour la recherche et l'innovation au niveau Européen. Ce programme s'articule autour de trois priorités : l'excellence scientifique, la primauté industrielle et les défis sociétaux. C'est dans le cadre de ce programme que le projet P4SB[3] vit le jour en avril 2015. Ce projet vise à produire des polymères à haute valeur ajoutée à partir des produits de la dégradation contrôlée de déchets plastiques par voie enzymatique. Pour cela, une souche de *Pseudomonas putida* doit être modifiée en utilisant notamment des outils de biologie synthétique.

Les matériaux plastiques ciblés dans ce projet sont le polyéthylène téréphtalate (PET) et les polyuréthanes (PU), respectivement les 5ème et 6ème matériaux plastiques les plus produits par an (PlasticsEurope 2017). Les PU, qui concernent le travail de ce mémoire, peuvent avoir des structures très variées ayant toutes en commun la présence d'une liaison uréthane (Figure I.1b). La diversité de structures répond à une diversité d'applications. Une large majorité des PU est synthétisée sous forme de mousses utilisées pour les secteurs de la construction ou de l'ameublement.

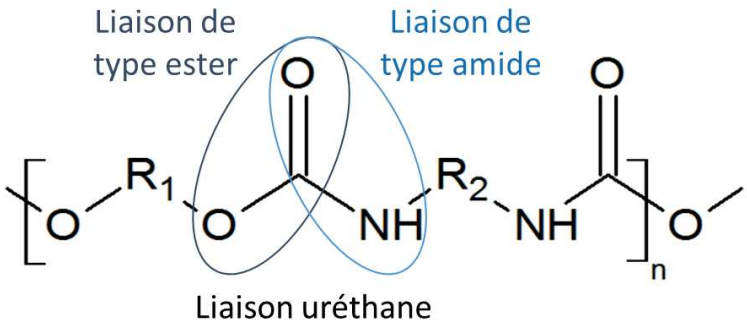

Figure I.2 – La liaison uréthane peut être vue comme la combinaison d'une liaison de type ester et d'une liaison de type amide

La première étape de ce projet est la dépolymérisation par voie enzymatique. Contrairement à celle du PET qui a été largement décrite (Herrero Acero et al. 2011; Ribitsch et al. 2015), la

[3] https://www.p4sb.eu/

dépolymérisation des PU est peu traitée dans la littérature. Des études ont montré la possibilité de dégrader certains types de PU mais les mécanismes sont encore mal connus et l'efficacité reste limitée à quelques structures chimiques (Cregut et al. 2013).

Suite à cette première étape, la modification des voies métaboliques du châssis bactérien de *Pseudomonas putida* représente un défi de grande ampleur. En effet, il convient d'apporter à cette souche la capacité d'assimiler les monomères issus de la dépolymérisation des déchets et d'optimiser sa capacité à produire et sécréter des produits d'intérêt, notamment des poly(hydroxyalcanoates) (PHA). Les PHA sont une famille de polyesters bactériens et biodégradables. Pour ces différentes étapes, des outils de biologie synthétique offrent des perspectives intéressantes (Figure I.3).

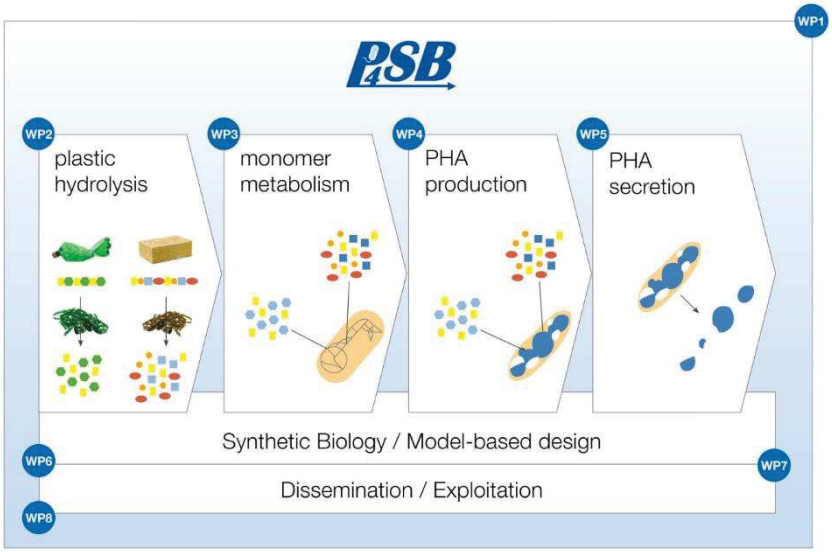

Figure I.3 – Workflow du projet P4SB

3. Déroulement de la thèse et organisation du manuscrit

Cette thèse s'est principalement déroulée dans l'équipe BioTeam[4], dirigée par le Pr. Luc Avérous, au sein de l'Institut de Chimie et Procédés pour l'Energie, l'Environnement et la Santé (ICPEES-UMR 7515 – Université de Strasbourg/CNRS). Les thématiques de recherche de la BioTeam

[4] http://www.biodeg.net/BioTeam.html

s'articulent autour des polymères biosourcés et/ou biodégradables pour des applications biomédicales et/ou environnementales.

L'Institut Charles Viollette (ICV), dirigé par le Pr. Pascal Dhulster, a été mon second laboratoire d'accueil. J'ai travaillé au sein de l'équipe Procédés Biologiques, Génie Enzymatique et Microbien (ProBioGEM) de l'ICV (Lille, France) avec le Professeur Vincent Phalip. La complémentarité entre les compétences de ProBioGEM et de la BioTeam m'ont permis d'avoir toutes les clés en main pour aborder d'une part les aspects de biochimie/microbiologie et, d'autre part, la chimie des polymères, requis pour la réalisation de cette thèse.

Cette thèse a été réalisée en étroite collaboration avec deux partenaires industriels du projet P4SB que sont Protéus (Nîmes, France, filiale du groupe Novacap), spécialisé en génie enzymatique, et SOPREMA (Strasbourg, France), leader mondial dans le domaine des membranes d'étanchéité du bâtiment.

Chaque chapitre de ce manuscrit est centré autour d'un article scientifique rédigé en anglais. Les articles sont encadrés par une introduction et une conclusion en français. Les chapitres peuvent aussi contenir une partie « résultats préliminaires » (avant l'article) et/ou une partie « résultats complémentaires » (après l'article). En effet, ces travaux ont donné lieu à de très nombreux résultats, qui n'ont pas été et ne seront pas tous valorisés par le biais de publications. Il nous a toutefois semblé important de les faire apparaitre dans ce manuscrit afin de mettre en lumière l'intégralité de la démarche scientifique adoptée. Les résultats préliminaires font parfois référence à des éléments de matériels et méthodes qui n'apparaissent que dans l'article du chapitre concerné. Nous nous sommes assuré que cela n'entravait pas la compréhension des résultats obtenus.

La dégradation biologique des polyuréthanes est une thématique originale. La réalisation d'un état de l'art bibliographique assez large sur cette thématique a donc été nécessaire (Figure I.4). A travers ce premier chapitre, nous avons notamment souhaité mettre en évidence la diversité des méthodes utilisées pour évaluer la dégradation des polyuréthanes. Cette étude a permis la mise au point de techniques utilisées dans la partie expérimentale de la thèse.

Dans un second chapitre, nous traitons de la dépolymérisation enzymatique des PU. Le développement et la réalisation d'un criblage d'une collection d'enzymes mise à disposition par Protéus sont ainsi décrits. Par la suite, les enzymes issues de ces criblages ont été caractérisées, en termes d'activité de dégradation, sur différents PU thermoplastiques (TPU) synthétisés par SOPREMA.

Le troisième chapitre traite de la dégradation fongique des PU. Une collection de souches de champignons a tout d'abord été constituée. Cette collection a ensuite été criblée sur des TPU. Les souches présentant les compétences de dégradation les plus importantes ont alors été identifiées et caractérisées plus en détail en ce qui concerne leur capacité à dégrader des PU.

Enfin, le quatrième chapitre propose une approche protéomique pour l'identification d'enzymes fongiques impliquées dans la dégradation des PU. Les protéines extracellulaires de deux souches d'intérêts sécrétées en présence de quatre substrats distincts ont ainsi été analysées. Cela permet de mettre en évidence des enzymes spécifiquement produites en présence de certains motifs structurels présents dans les substrats testés.

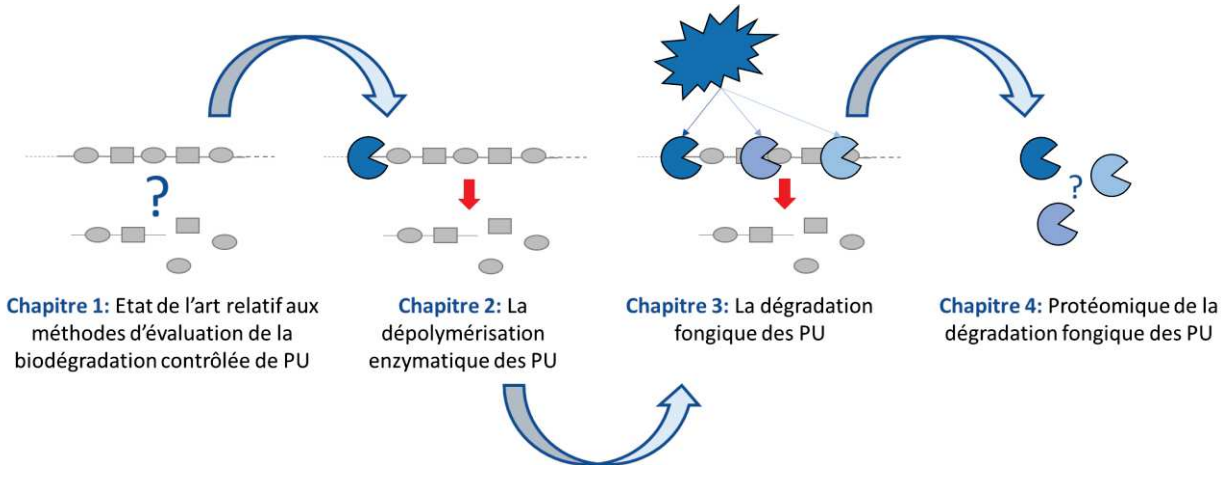

Figure I.4 – Schéma récapitulatif de l'organisation des travaux de thèse dans ce mémoire.

4. Références

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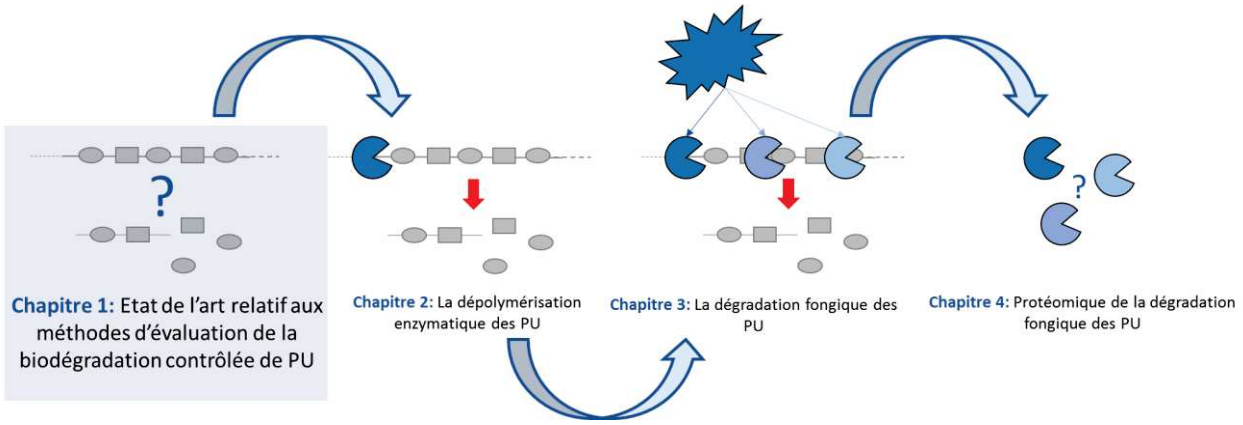
Chapitre 1. Etat de l'art relatif aux méthodes d'évaluation de la biodégradation contrôlée de polyuréthanes



Introduction

Les objectifs de cette thèse concernent la dégradation enzymatique et microbienne d'un large panel de polyuréthanes (PU). En fonction de l'entité biologique ciblée (enzyme ou microorganisme) et du type de substrat PU, les tests de dégradation et les techniques implémentées varient grandement. La première étape expérimentale de ce travail de thèse fut de développer des méthodes permettant d'évaluer les performances de dégradation d'enzymes et de microorganismes sur les substrats PU. Pour soutenir cette première étape, une synthèse bibliographique discutant des différentes techniques d'évaluation de la dégradation biologique des PU a donc été réalisée.

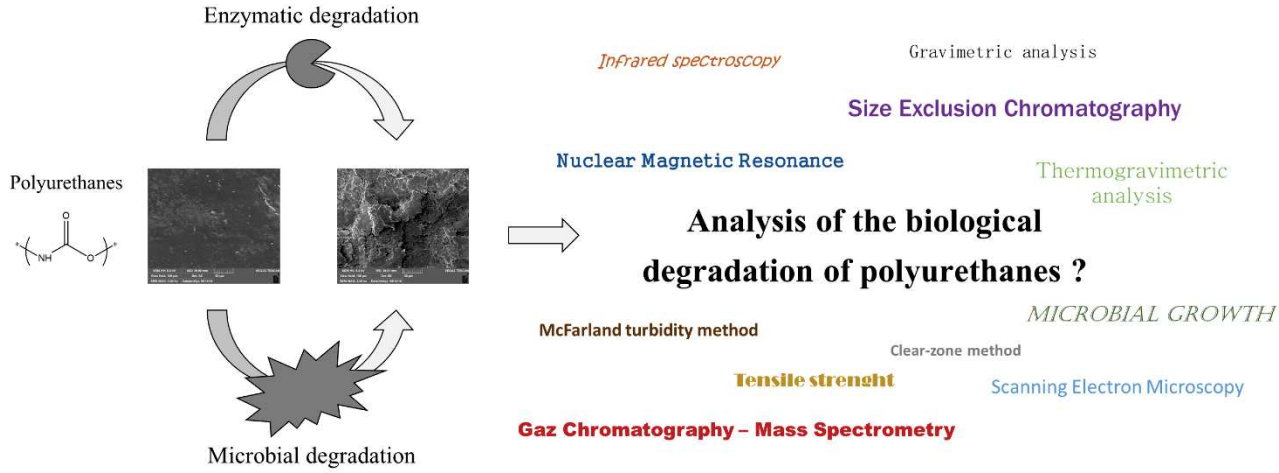
La dégradation biologique des polyuréthanes est un sujet transdisciplinaire faisant appel à des connaissances spécifiques en chimie (des polymères et analytique) ainsi qu'en biologie (microbiologie et biochimie). Au cours de cette synthèse bibliographique, nous avons souhaité apporter les définitions nécessaires à la compréhension du sujet dans sa globalité pour un lecteur non spécialiste de cette thématique.

Polyurethane biological degradation: A technical overview

1. Abstract

Polyurethanes (PU) are a family of versatile synthetic polymers intended for diverse areas and applications. The biological degradation of PU is a hot-topic as it contributes to the development of green recycling process and to the design of eco-friendly materials. Indeed, an increasing number of studies deal with the discovery and characterization of enzymes and micro-organisms able to degrade PU. The synthesis of short lifespan PU material sensitive to biological degradation is also of growing interest. Measurement of PU degradation can be performed by a wide range of analytical tools depending on the nature of the material, on the biological degrader and on the purpose of the experiment. Recent evolutions of these techniques and approaches allowed a better understanding of the mechanisms involved in PU biodegradation. This review offers an overview of the technical solutions implemented for the analysis of PU biological degradation. Advantages, drawbacks, specific use and output of these methods are discussed to provide a critical overview of this topic.

Keywords: Microbial degradation, enzymatic degradation, polyurethanes, technical review



2. Introduction

Despite the comfort provided by plastic materials to our everyday life, they are cause of global and increasing pollution. Indeed, their massive production involves a polluting exploitation of fossil resources and their poor waste management induces environmental contamination. For instance, in 2010, more than 275 million metric tons of plastic waste were generated in about 200 coastal countries of which from 5 to 13 million metric tons are estimated as reaching the oceans (Jambeck et al. 2015). Consequently, plastic wastes accumulate in this environmental compartment. With the problematic of the nano or microplastics debris, the ocean garbage patches are major environmental concerns of this century (Law et al. 2010; Eriksen et al. 2013; Cozar et al. 2014). Even if some studies still question the actual impact of plastic waste (Duis and Coors 2016), many others are warning of irrevocable environmental damages (Clukey et al. 2018; Darmon et al. 2017; Galloway and Lewis 2016). By entering the food chain, plastic materials attain human causing health concerns (Chae and An 2017; Bouwmeester, Hollman, and Peters 2015; Barboza et al. 2018).

Among the vast families of resistant plastic materials are the polyurethanes (PU). Their presence in marine ecosystems has been attested (Reddy et al. 2006; Frère et al. 2016). In 2016, Turner at al. reveals that over the 70 foamed plastics fragments collected on a Britain beach, 39 were identified as PU (Figure 1.1), thus pointing out the impact of PU in plastic pollution (Turner and Lau 2016).

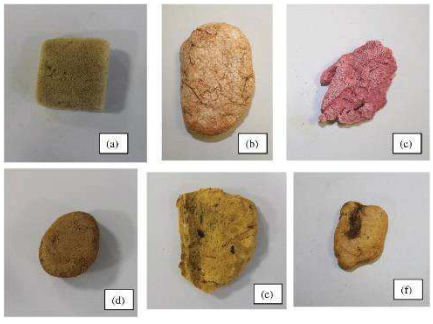


Figure 1.1 – Foamed plastic debris collected on a Britain beach, pieces a, b d, e and f are PU, adapted from Turner et al., 2016 (Turner and Lau 2016)

Firstly synthesized in the 1930s by the German chemist Otto van Bayer, PU products were commercialized about 10 years later (Bayer 1948). The use of PU spreads during the World War II, where it replaced natural rubber for elastomer production. Rapidly, other applications in aviation and textile, of which were flourishing fields during this period, emerged. Diversification of PU properties allowed to reach numerous markets. In 2016, 18 million tons of PU were produced worldwide, representing 5.3% of global plastic production (Furtwengler, Boumbimba, and Avérous 2018; PlasticsEurope 2017). PU rank at the 6th most produced synthetic polymers. About 22 million tons are expected to be produced in 2020 (Akindoyo et al. 2016).

The most widespread form of PU is foams. In 2014, the PU foam market was valued at $46.8 billion and is expected to reach $72.2 billion by 2020 (Pillai et al. 2016). Flexible and rigid foams represent respectively 32% and 36% of the global PU production. Flexible foams, renowned for their comfort, are used for the cushioning of furniture, bedding or for automotive seats. In the construction area, rigid foams are preferred for thermal insulation on agreement with a growing demand for energetically efficient buildings.

PU are also widely used as coatings, adhesives, sealants and elastomers (CASE). PU coatings provide a protection layer against weather, abrasion and corrosion. Elastomers are elastic and flexible and can adopt any desired shape. In the medical field, PU elastomers are used for the development of cardiovascular devices, orthopedic prosthesis and nerve regeneration (Gunatillake, Adhikari, and Felton 2011). Vectors for drug delivery based on biocompatible polyurethanes are also increasingly considered (Zhou et al. 2012).

The common thread between most of these PU materials is that they are mostly intended for long-term applications. They are thus designed to resist environmental factors such as microbial degradation, abrasion, moisture or UV.

Biodegradation can be generally defined as the decomposition/degradation of materials by the means of biological entities such as micro-organisms or enzymes. This process is used for numerous industrial applications such as waste water treatment (Watanabe 2001) or depollution of site contaminated by e.g., polycyclic aromatic hydrocarbons (Shuttleworth and Cerniglia 1995). Polymer degradation by micro-organisms occurs in several phases (Figure 1.2). First, materials are

fragmented into pieces thanks to abiotic and biotic factors such as UV, abrasion or biofilm formation in biodeterioration steps. Then, macromolecules are cleaved by enzymatic hydrolysis and/or oxidation, leading to the release of low molar mass molecules, oligomers and monomers. These molecules are finally assimilated and mineralized by microorganisms to promote microbial growth (Lucas et al. 2008). A countless number of mineralization paths exists in nature.

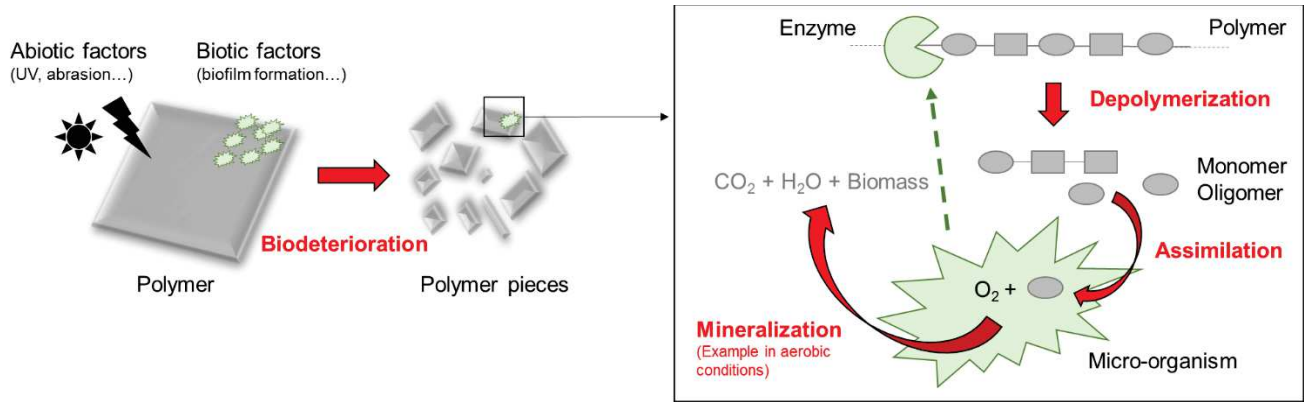


Figure 1.2 – Polymer biodegradation process

The step of enzymatic depolymerization or enzymatic degradation can be reproduced *in vitro,* independently of the microbial degradation. Interestingly, the released molecules resulting from enzymatic depolymerization of synthetic polymers could be turn into building blocks and high value products for the chemistry market (Wierckx et al. 2015).

PU are not biodegradable polymers (Wierckx et al. 2018). Even if some PU can be sensitive to biological degradation, they do not answer requirements of the European norm EN 13432 defining biodegradable and compostable materials (Avérous and Pollet 2012). This norm considers a material as biodegradable if the degradation reaches 90% after 6 months under composting conditions. The non-toxicity of the degradation products is also a requirement of this norm.

Biodegradation of PU has been studied since the 1960s. The first publications on this topic aimed to evaluate the microbial susceptibility of PU formulations in order to promote the development of highly resistant materials (Darby and Kaplan 1968; Kaplan et al. 1968; Kanavel, Koons, and

Lauer 1966; Edmonds and Cooney 1968; Cooney 1969). However, this trend must be reversed after use. Due to increased environmental concerns, sensitivity to microbial degradation has become a desired feature to reduce the environmental footprint of short-term application PU materials (Prieto 2016). Meanwhile, the development of bioresorbable PU material for the biomedical industry raised (Špírková et al. 2017; Pavlova and Draganova 1993). Currently, PU biodegradation assessments are focused on two main purposes: (i) biodegradation susceptibility of new eco-materials or materials intended for biomedical purpose, thus focusing on the polymer synthesis and (ii) bioremediation or biological recycling of PU, thus focusing on the biological agents capable of degradation.

Techniques used to evaluate the biodegradation of PU are very diverse. Depending on the type of PU, on the degrader and on the goal of the experiment, techniques implemented will differ. High variability of interpretation is sometimes observed in-between publication leading to confusing results and conclusions. Discussing the techniques and their output helps setting up methods and concluding on results. Significant advances have recently been made on the evaluation of PU biodegradation and consequently, on the understanding of degradation mechanisms.

Reviews recently published on PU biodegradation have mainly oriented their discussions towards degrading micro-organisms (Mahajan and Gupta 2015), degrading enzymes (Loredo-Treviño et al. 2011) or PU biodegradation for recycling (Cregut et al. 2013). To complete this set, our review aims at providing a comprehensive overview of the advances on PU biodegradation focusing on how it can be evaluated. The first part is dedicated to the diversity of PU: compositions, structures and waste management are addressed. Then, the biological agents able to degrade PU are presented. Finally, the technical solutions used to assess PU biodegradation are gathered. Their uses, advantages and drawbacks are discussed.

3. Polyurethanes structures and biological susceptibility relationships

3.1. Effect of the composition and structure

Chemical composition and macromolecular architecture are of importance for the biodegradation of polymers (Kim and Kim 1998). The nature of the chemical bonds, crystallinity and molar mass are key parameters influencing the polymer susceptibility to biological attack (Zeng et al. 2016). PU are characterized by the urethane or carbamate linkage, generally obtained by addition of an isocyanate and a hydroxyl group (Figure 1.1Figure 1.3). For instance, in thermoplastic PU (TPU) synthesis, a polyisocyanate reacts generally with a polyol, principally long polyester- or polyether-based polyols, with a controlled functionality closed to 2. A linear prepolymer with isocyanate ending groups is formed. Then a chain extender, usually a short diol, is added to obtain high molar mass polymer.

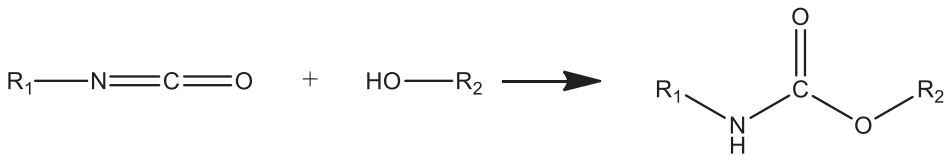


Figure 1.3 – Polyaddition of an isocyanate and a hydroxyl group to form an urethane bond

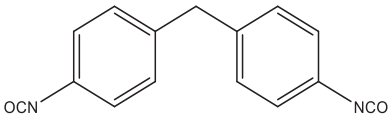
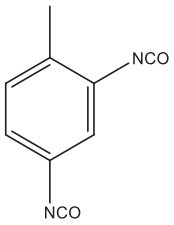
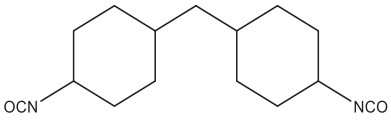
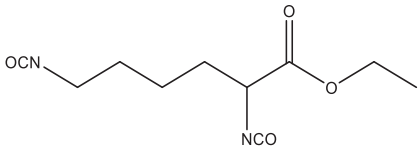
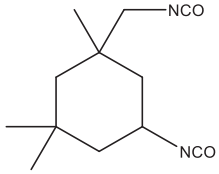
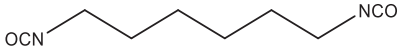
Most frequently used isocyanates are bifunctional aromatic molecules: 4,4’ methylene diphenyl isocyanate (4,4’ MDI) and toluene diisocyanate (2,4 TDI) respectively involved in rigid and flexible polymer production (Table 1.1) (Delebecq et al. 2013). Thanks to its double aromatic ring, MDI is the most used isocyanate for rigid foams, one of the most prevalent polyurethane-based market (Sabbioni et al. 2012).

To a lesser extent, aliphatic isocyanates are also of interest such as isophorone diisocyanate (IPDI), hexamethylene diisocyanate (HDI), lysine diisocyanate (LDI) or 4,4'-methylene dicyclohexyl diisocyanate (H_{12}MDI) (Table 1.1). They cope with aromatic diisocyanate limits. They are also preferred for medical devices because of the mutagenicity of diamine derived from aromatic diisocyanate hydrolysis (Darby, Johnson, and Northup 1978). For the formulation of waterborne

polyurethane dispersion aliphatic isocyanate are chosen because aromatic ones are more reactive with water and thus make the synthesis hard to handle (Noble 1997). Finally, in the frame of sustainable PU materials development, non-isocyanate polyurethane (NIPU) is a hot topic. Indeed, isocyanates derived from phosgene are extremely toxic and pollutant compounds. NIPU can be obtained by reaction between amines and cyclic carbonates to form polyhydroxy-urethanes (Carré, Bonnet, and Avérous 2015).

The high reactivity of isocyanates makes them unstable in water. Consequently, even if free isocyanates remain on the polymer after synthesis, the incubation in aqueous media for biodegradation will immediately neutralized these bonds leading to amine or urea moieties. No isocyanate can be found as degradation products after degradation assay.

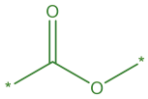
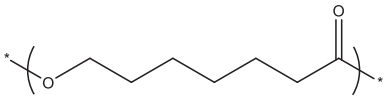
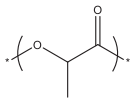
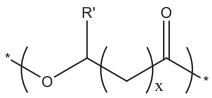
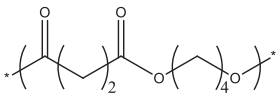
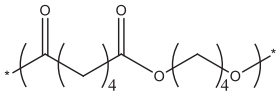
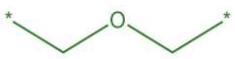
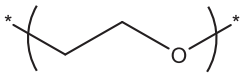
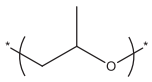
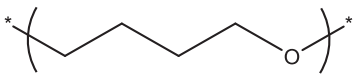
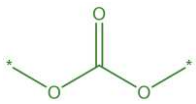
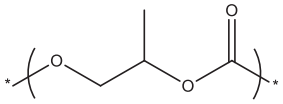
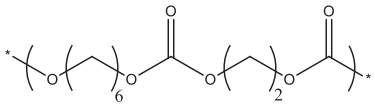
Table 1.1 – Structure of the most common isocyanates

IUPAC name	Abbreviation	Type	Structure	Reference
1-isocyanato-4-[(4-isocyanatophenyl)methyl] benzene	4, 4' MDI	Aromatic		(Shah et al. 2016)
2,4-diisocyanato-1-methyl-benzene	2, 4 TDI	Aromatic		(Spontón et al. 2013)
1-Isocyanato-4-[(4-isocyanatocyclohexyl) methyl]cyclohexan	H_{12}MDI	Aliphatic cyclic		(Brzeska et al. 2015)
Ethyl Ester L-Lysine Diisocyanate	LDI	Aliphatic linear		(Zhou et al. 2012)
5-isocyanato-1-(isocyanatomethyl)-1,3,3-trimethyl-cyclohexane	IPDI	Aliphatic cyclic		(Pereira et al. 2012)
1,6-diisocyanatohexane	HDI	Aliphatic linear		(Tang, Labow, and Santerre 2001)

One of the main components of PU synthesis is the polyol. Common polyols can be polyether, polyester or more rarely polycarbonates. Higher flexibility of polyether polyols make them more conventional for polyurethane production (Krasowska et al. 2012). A non-exhaustive list of polyols

with their structures is available in Table 1.2. Polyols ordinarily used are fossil-based molecules but an increasing number of studies deal with bio-based polyols, on agreement with a greener chemistry. Compounds from vegetal sources such as castor oil (Trovati et al. 2010), starch (Duarah et al. 2016) or aromatic polymers such as tannins or lignin (Ignat et al. 2011) are increasingly incorporated in PU formulation.

Table 1.2 – Examples of polyols used for PU synthesis

Polymer name	Abbrev.	Structure	Reference
Polyester			
Poly(caprolactone)	PCL		(Yeganeh and Hojati-Talemi 2007)
Poly(lactic acid)	PLA		(Izadi-Vasafi et al. 2017)
Poly(hydroxyalkanoates)	PHA		(Pérez-Lara et al. 2016)
Poly(butylene succinate)	PBS		(Li et al. 2015)
Poly(butylene adipate)	PBA		(Shah, Krumholz, et al. 2013)
Polyether			
Poly(ethylene glycol)	PEG		(Zhang et al. 2013)
Poly(propylene glycol)	PPG		(Chattopadhyay et al. 2008)
Poly(tetramethylene glycol)	PTMEG		(Wiggins, Anderson, and Hiltner 2003)
Polycarbonate			
Poly(propylene carbonate)	PPC		(Chen et al. 2016)
Poly(1,6-hexyl 1,2-ethyl carbonate)	PHEC		(Christenson, Anderson, and Hiltner 2004)

In the case of e.g. TPU synthesis, chain extenders are used. They are mainly low molar mass diols such as 1,4-butanediol, ethylene glycol, 1,6-hexanediol (Akindoyo et al. 2016). Low molar mass diamine can also be used such as ethylene diamine (Tang et al. 1997), thus creating urea instead of urethane when reacting with isocyanate. These chain extenders have a significant impact on the polyurethane structure and properties through phase separation of soft and hard segments.

Hard segment, which is the highly ordered region, is a block segment formed from the isocyanate and the chain extender while soft segment, or the amorphous region, is mainly the long polyol part (Figure 1.4). Hard segment content and chemistry have already shown influence on the biodegradation susceptibility of a polycarbonate PU (Tang, Labow, and Santerre 2003a; Tang, Labow, and Santerre 2001).

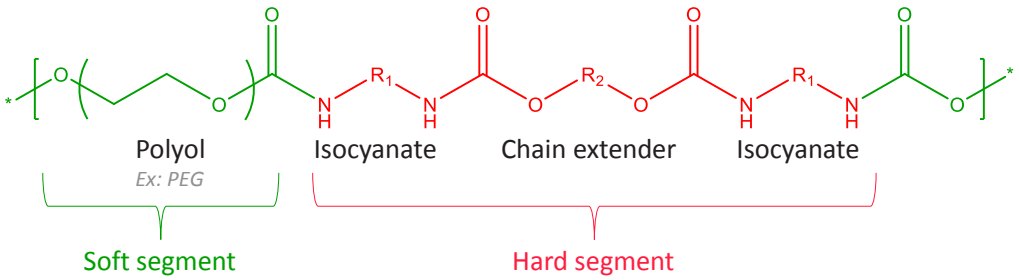


Figure 1.4 – Linear polyurethane structure

PU, as many other polymers, can be synthesized as thermoplastics or thermosets. Thermoplastics are linear or slightly cross-linked/branched structures. Isocyanates, polyols and chain extenders used for TPU synthesis have only two functional groups (diols, diisocyanates …). Thermoplastics can be melted or present a liquid-like behavior when increasing the temperature without polymer denaturation. By contrast, thermosets are highly cross-linked polymers with 3D structure induced by molecules with a functionality higher than 2. Due to their architecture, thermosets do not melt and are denatured at high temperature and thus cannot be reshaped by heating unlike thermoplastics.

PU foams are thermoset materials. The alveolar structures of these complex systems are obtained with blowing agents (air, carbon dioxide, hydrocarbons such as isopentane or other gaseous

substances). Foams are structured by strut and walls defining cell cavities. Cells can be closed (closed-cell foam, mainly rigid) or open (open-cell foam, mainly soft) (Gautam, Bassi, and Yanful 2007a). Type of cells highly impact the foam properties. Side reactions during foaming induce the formation of various reversible and irreversible bonds. It is important to consider the bonds nature when dealing with foam degradation. Because of the variety of bonds, it is difficult to know if the urethane bond is involved after degradation. Commercial foams are supplemented by additives such as fire retardants, antioxidant or anti-microbial compounds that can prevent microbial degradation.

Because of the nanometric size of particles and their hydrophilicity, waterborne PU dispersions (WPUD) are particularly suitable for biological assays. WPUD are synthesized using an emulsifier. In the case of dimethylpropionic acid (DMPA), two functional hydroxyl groups react with the isocyanates (Figure 1.5). The hydroxyl group of the carboxylic acid does not react with isocyanate because of steric hindrance and lower reactivity (Coutinho, Delpech, and Alves 2001). Hydrophilic carboxylic acid then forms a top-layer around the hydrophobic polymer.

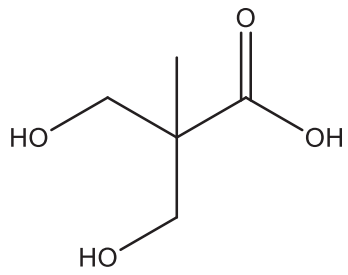


Figure 1.5 – Structure of the dimethyl propionic acid (DMPA)

3.2. PU waste disposal

The diversity of PU macromolecular structures and relative chemistry (described above) is an obstacle for efficient waste management. To appreciate the importance of PU biodegradation, it is necessary to analyze the current PU waste disposal. PU waste are post-consumer products as well as production waste, mainly from foam. Indeed, scrap from slabstock can reach up to 10% of the production (Simon et al. 2018). In France, scrap PU were estimated at 13 kt in 2011 while the

general end-of-life products volume is about 198 kt (Boujard, Foray, and Caudron 2014). This source of PU waste mainly arises from construction, furniture and bedding, automotive, shoes and home appliance. Efficient collect and product dismantling are required to recycle these materials thus limiting their valorization.

PU wastes are mainly treated by three ways: landfilling, incineration (which can also be considered as quaternary recycling (Ignatyev, Thielemans, and Vander Beke 2014)) and conventional recycling. Landfill discharge is still the main option but is gradually decreasing since this practice requires large land areas and no value is made of the waste. Landfilling and the absence of waste management lead generally to pollution.

Incineration presents the advantage of being a mature technique, practiced for several decades. Energy is recovered by burning waste and can totally or partly, offset the energy spent in the heating process. Because of the low value recovered it is hardly considered as recycling. It is a solution to reduce landfill volumes, yet, incineration is not a satisfying strategy as it may lead to the release of toxic gasses in the atmosphere (Paabo and Levin 1987).

Depending on the nature (thermoplastics vs. thermosets), recycling processes differ. TPU can be heated and remolded thus making physical recycling process easier. However, only about 1% of PU are estimated to be recycled thanks to physical methods (Behrendt and Naber 2009). The recycling of PU foams is more challenging since it cannot be remolded. The main path for foam recycling is regrinding. Nearly 500 000 tons of recycled PU was used in 2010 for carpet underlay[5].

Chemical recycling can address both thermoplastics and thermosets architectures (Wang et al. 2011; Simón et al. 2016). Depending the polyol part structure, glycolysis appears as the most promising techniques (Simon et al. 2018). It is a transesterification reaction: the ester group of the urethane bond is interchanged by the hydroxyl group of a diol (glycol) added in large excess (Simón et al. 2013). Simon et al., developed a glycolysis process allowing polyether recovery from high resilience PU foams (Simón et al. 2016). These polyols can then serve as building blocks for synthesis of second-generation polymers. The major limit of chemical recycling is the processing

[5] https://polyurethane.americanchemistry.com/Polyurethane-Recycling/

temperature that lead to high energy consumption. For instance, in the above-mentioned example, temperature of glycolysis is 190°C.

Biological recycling is a growing route that might answer the need for PU recycling in the coming years. This is a soft process that can be implemented at low temperature (less than 70°C) (Mueller 2006; Valerio 2010). This process is catalyzed by biological agents, either micro-organisms or enzymes. The resulting degradation products may then be valorized. Efficient enzymatic depolymerization of poly(ethylene terephthalate) (PET) has been demonstrated at 60°C leading to the release of valuable building blocks (Gamerith et al. 2017). This result, based on PET, can be consider as a first benchmark for PU.

4. Actors of the biodegradation step: Biological entities and associated mechanisms

4.1. Microorganisms

Biodegradation involving micro-organisms can be performed by a consortium (mixed culture) or a single strain (Figure 1.6). Microorganisms have the ability to form biofilm on the surface of polymers by adhesion (Sivan 2011). Once colonized, the material may appear as a source of carbon and nitrogen thus promoting microbial growth.

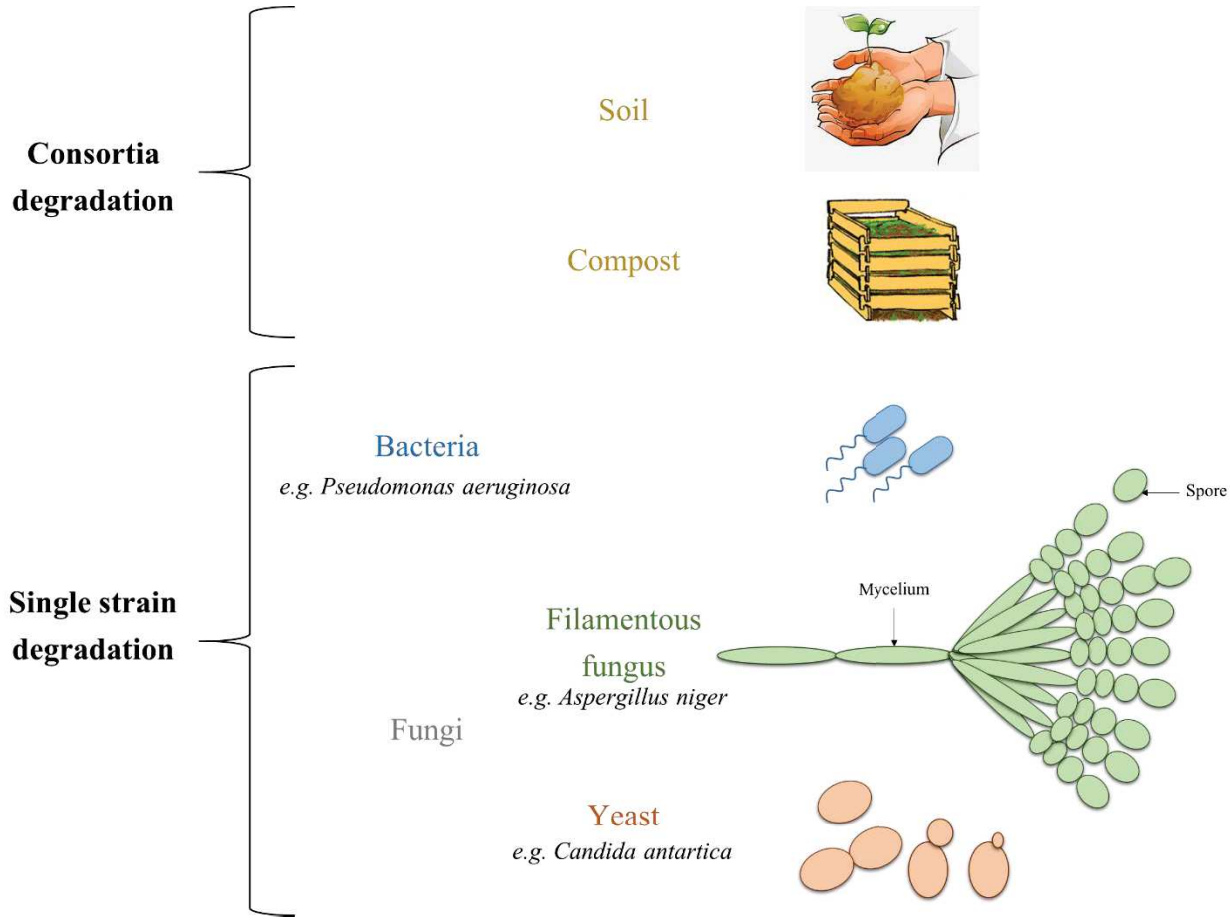


Figure 1.6 – Different types of microbial PU degraders

4.1.1. Microbial consortia

A consortium is a group of two or more micro-organisms living symbiotically or living in a mixed culture *in vitro* because of complementary activities. The main advantage of a consortium is that each member of the consortium can bring complementary degradation pathways. Comparison of degradation skills of a strain of fungi (*Aspergillus niger*) and a strain of bacteria (*Pseudomonas aeruginosa*) revealed a slightly higher TPU degradation with the bacteria but, above-all, an impressive synergistic effect when the polymer was incubated with both strains (Fernandes et al. 2016). Composting and soil burial degradation are often performed on long time scale going from 28 days (Bentham, Morton, and Allen 1987) to one or two years of incubation (Gómez et al. 2014; Lu et al. 2016; Zhang et al. 2013; Krasowska et al. 2012).

Temperature plays an important role in composting processes. It can reach up to 58°C (Genovese et al. 2016) while degradation with bacteria or fungi are generally performed between 25 and 37°C. High temperatures favor polymer degradation due to the increase in polymer chain mobility and thus an easier access to potentially cleavable bonds.

Undefined consortia are also convenient for the discovery of new degrading micro-organisms. After some times of incubation with a consortium, a selection of strains occurs naturally: micro-organisms unable to consume the polymer die in favor of competent organisms that are able to use the polymer as a substrate for their own growth. Isolation and identification of remaining micro-organisms can further be performed (Barratt et al. 2003). For this purpose, samples are generally collected in environments already acclimated to the presence of synthetic polymer. For instance, Rattanapan *et al*. collected an inoculum in sewage water of a rubber latex factory (Rattanapan et al. 2016) whereas Cregut *et al*., collected inoculum for degradation experiments in an industrial PU foam plant site (Cregut et al. 2014). In this last publication, acclimation of the inoculum is performed by successive exposure to PU.

Another option is to collect strains from extreme media as it was the case for Russell *et al*., who isolated endophytic fungi from wood collected in the Ecuadorian Amazonian rainforest. This hot and humid environment is well-known for the richness of its biodiversity (Russell et al. 2011). Isolated strains are highly adapted to the degradation of recalcitrant vegetal biomass and are thus inclined to have degradation activity on recalcitrant synthetic polymers.

4.1.2. Fungi

It is possible to appraise only the fungal activity of a consortia or isolate only fungal strain by adding an antibiotic which prevent bacterial growth. For instance, 50 g/ml chloramphenicol can be added to the medium to prevent bacterial growth and allow analyzing only strains of fungi (Zafar, Houlden, and Robson 2013)

Micro-organisms from the fungi kingdom described as PU degraders are almost only filamentous fungi. Only two studies on yeast were found: one dealing with a yeast displaying *Candida antarctica* lipase B (CALB), a highly active enzyme, on its surface (Shibasaki et al. 2009) and

another describing the growth of *Cryptococcus sp.* MTCC 5455 on fish waste to produce a lipase showing activity on PU (Thirunavukarasu et al. 2015).

A general review on fossil-based polymers biodegradation testing interestingly gives significant importance to abiotic aspects of fungal biodegradation (Lucas et al. 2008). The formation of filaments exerts physical pressure leading to polymers breaking. Filament apices penetrate in the material increasing the size of pores and provoking cracks. Both fungi and bacteria were isolated from biofilms formed after soil burial of a polyester PU (Barratt et al. 2003). Evaluation of their degradation skills revealed that fungi are more involved in the biodegradation in soil than bacteria.

Five strains are recommended by the American Society for Testing and Materials (ASTM) to evaluate the fungal resistance of material: *Aspergillus niger* ATCC 9642, *Aureobasidium pullulans* ATCC15233, *Chaetomium globosum* ATCC6205, *Gliocladium virens* ATCC9645, *Penicillum pinophilum* ATCC11797 (ASTM code: G21 - 90 Standard Practice for Determining Resistance of Synthetic Polymetric Materials to Fungi) (Gu and Gu 2005). For instance, Oprea et al., developed new eco-friendly biomaterials and tested their fungal resistance with a strain *Chaetomium globosum* from 90 to 130 days (Oprea and Doroftei 2011; Oprea 2010; Oprea et al. 2016).

Strains belonging to the genus of *Alternaria* (Matsumiya et al. 2010; Oprea et al. 2018), *Aspergillus* (Khan et al. 2017; Osman et al. 2018; Mathur and Prasad 2012), *Cladosporium* (Alvarez-Barragan et al. 2016) are frequently isolated from environmental samples. Fungal degradation has been demonstrated on both polyester and polyether-based PU, on TPU, foams and dispersion. However, mechanisms of degradation are not clearly elucidated. The part of biotic and abiotic factor in fungal degradation still need to be clarified.

4.1.3. Bacteria

Bacteria are mainly studied toward the degradation of TPU or PU dispersion. Cooney et al., described the bacterial degradation of a polyester-based PU foam by *Pseudomonas aeruginosa (Cooney 1969)*. As far as we know, it is the unique publication dealing with bacterial degradation of foams.

Pseudomonas is probably the most studied genius. The strain of *P. aeruginosa* ATCC 13388 is the only recommended strain by the ASTM for testing material resistance to bacterial degradation (ASTM code G22-76: Standard Practice for Determining Resistance of Plastics to Bacteria) (Gu and Gu 2005; Kay, Morton, and Prince 1991). Gogoi et al., used this strain to evaluate the bacterial susceptibility of bio-based waterborne PUD (Gogoi and Karak 2017; Gogoi and Karak 2015, 2014). Other *Pseudomonas* species were described as PU degraders (Biffinger et al. 2015; Peng et al. 2014; Gautam et al. 2007). Furthermore, *Pseudomonas* strains are known to be of high interest in white biotechnology (Wierckx et al. 2015). Numerous metabolic pathways of interest have been evidenced within this genus for bioremediation purposes. For instance, *P. putida* is a poly(hydroxyalkanoate) (PHA) producer (Poblete-Castro et al. 2012) and *P. aeruginosa* is a rhamnolipid producer (Rahman et al. 2002). PHA is a biodegradable polyester produced by bacterial fermentation whereas rhamnolipids are bio-surfactants.

Crookes-Goodson et al., evaluate the relative contribution of biofilm versus planktonic cells of *P. fluorescens* to PU degradation (Crookes-Goodson et al. 2013). Biofilm formation seems to accelerate the degradation process thanks to the secretion of enzymes in close contact with PU. They also highlighted the formation of intracellular granules under nutrient limitation conditions. This stationary phase affects the degradative capacity of the biofilm.

Strains of *Bacillus* such as *Bacillus subtilis* (Shah et al. 2016), *B. pumilus* (Nair and Kumar 2007) and *B. amyloliquefaciens* (Rafiemanzelat, Jafari, and Emtiazi 2015) are also isolated from soil or PU-contaminated water for their degradation competencies. *Rhodococcus equi* strain TB-60 particularly drawn interest because of its ability to hydrolyze the urethane bond from model molecules (Akutsu-Shigeno et al. 2006; Kambe and Shigeno 2009).

4.2. Enzymes

Enzymes are biological catalysts. Enzymes identified as PU degraders are originated from micro-organisms but also from mammalian cells such as lipase from porcine pancreas (Ng et al. 2017) or from plants such as papain from *Carica papaya* (Ferris et al. 2010). Enzymes used for PU degradation assays are either commercial enzymes or enzymes identified in PU-degrading micro-organisms which were then cloned, over-expressed in model micro-organisms and sometimes

purified. Reaction can occur at higher temperature than for bacterial or fungal degradation. Thermostable enzymes are described with an optimal degradation temperature of 70°C (Schmidt et al. 2017).

Enzymatic degradation assays on PU using oxidase enzymes (EC 1): horseradish peroxidase (EC 1.11.1.7), xanthine oxidase (EC 1.2.3.2) cytochrome C oxidase (EC 1.9.3.1) have been tested without success (Santerre, Labow, and Adams 1993; Santerre et al. 1994; Smith, Williams, and Oliver 1987). Hydrolytic enzymes seem to be the only class of PU degrading enzymes.

Several hydrolytic mechanisms have been highlighted for PU degradation. The most common is the hydrolysis of the polyester part of polyester-based PU by esterases (EC 3.1). Ester hydrolysis leads to the release of a carboxylic acid and an alcohol (Figure 1.7a). Esterases such as lipases (EC 3.1.1) (Schöne et al. 2016; Fang et al. 2014), cutinases (EC 3.1.1.74) (Yang et al. 2013; Schmidt et al. 2017) or unspecific esterases (EC 3.1) (Kang et al. 2011) have been described.

Urethane bond is often described as cleavable by esterase leading to carbamic acid and alcohol chain-ends (Wei and Zimmermann 2017; Mahajan and Gupta 2015). However, this mechanism does not seem conceivable because of the instability of the carbamic acid which immediately breaks down into an amine with the release a molecule of carbon dioxide (Ionescu 2005; Ozaki 1972) (Figure 1.7b).

Most of the implemented analyses concern polyester-based PU and do not allow differentiation between ester and urethane bond hydrolysis. The best way to evaluate the urethane bond hydrolysis by an esterase is thus to perform an assay on a substrate that does not contain ester bonds. Only few publications describing slight esterase activity on polyether PU show the potential ability of esterase to hydrolyze the urethane bond (Smith, Williams, and Oliver 1987; Santerre et al. 1994). Recently, a cholesterol lipase was reported to display activity on a PU based on triethylene glycol and 1,4-di-S-benzyl-D,L-dithiothreitol (Ferris et al. 2010). Urease (EC 3.5.1.5) showed activity on a poly(ether urea) PU (Phua et al. 1987) but the degradation is mainly attributed to the urea bond hydrolysis (Figure 1.7c).

Amidases (EC 3.5.1.4) and proteases hydrolyze amide or peptidic bond leading to the release of a carboxylic acid and an amine (Figure 1.7d). These enzymes appeared to be also efficient for the

hydrolysis of the urethane bond leading to the release of an amine, an alcohol and carbon dioxide (Figure 1.7e). Proteases such as papain (EC 3.4.22.2) (Yamamoto et al. 2007; Campinez et al. 2013; Ferris et al. 2010), bromelain (EC 3.4.22.32/33) (Yamamoto et al. 2007), ficain (EC 3.4.22.3) (Yamamoto et al. 2007) and chymotrypsin EC 3.4.21.1 (Ciardelli et al. 2004; Elliott et al. 2002; Ferris et al. 2010) are also widely described for the degradation of PU.

An amidase recently drew attention. This enzyme was isolated from *Nocardia farcinica* with the specificity of being able to hydrolyzed polyamides (Guo et al. 2014) but also both the ester and amide bonds of non-water soluble model substrates (Heumann et al. 2009) and polyester-based PU (Gamerith et al. 2016).

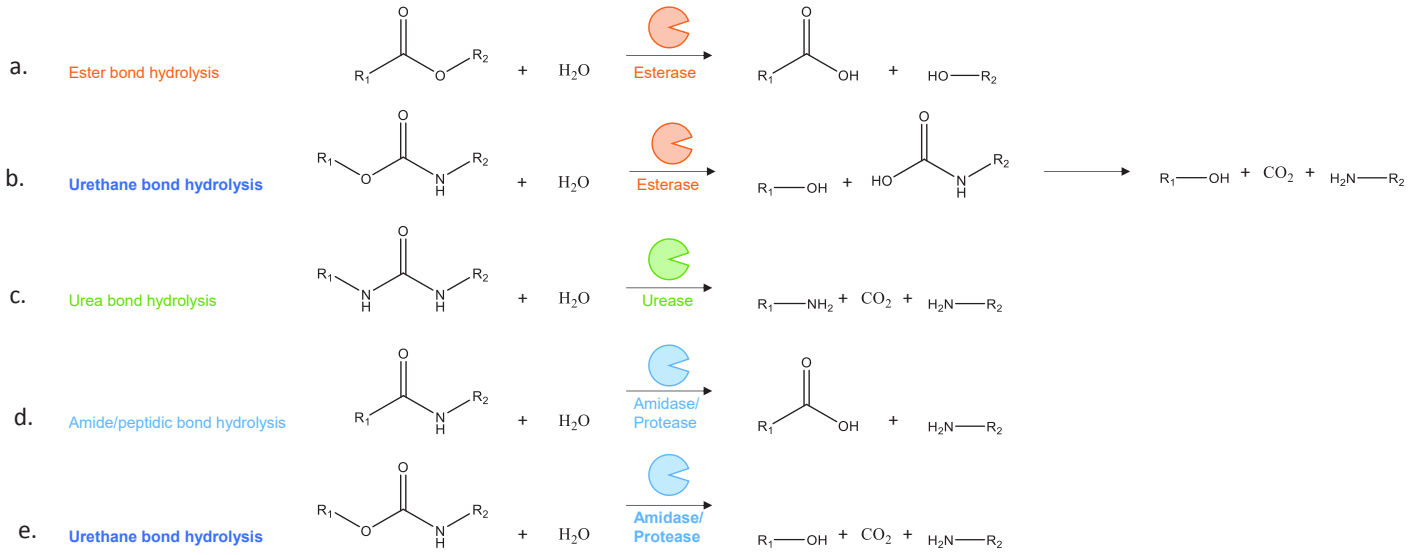


Figure 1.7 – Mechanisms of enzymatic degradation of PU

Up to now, PU depolymerization by enzymes is not efficient enough for the development of recycling processes. Recent publications on this topic oriented the research towards the improvement of the reactions.

Novel strategy to discover efficient enzymes was recently published. Metagenomics tools allowing to screen bovine rumen microbiota was developed to select enzymes with activity toward carbamate insecticides and PU (Ufarte et al. 2017). The main advantage is the possibility

of studying enzymes from uncultivable microorganisms and thus having access to new degradation skills.

Enzyme complementarity presented above justifies testing cocktail of enzymes with different activities. However, such trials have been performed between an elastase and an esterase without success (Ozsagiroglu, Iyisan, and Guvenilir 2012).

Synthetic biology is also a strong path for enzymatic degradation improvement. This approach already proved itself on PET-degrading enzymes. As an example, decreased sensitivity of a PET-degrading cutinase to an inhibitor has been successfully achieved by amino-acid modification into the catalytic site (Wei et al. 2016). Ribitsch et al., fused a PET-degrading enzyme (a cutinase from *Thermomyces cellullosylitica*) with a binding module of a PHA depolymerase from *Alcaligenes faecalis* (Ribitsch et al. 2013) to improve enzyme/polymer interactions. This binding domain was recently added to the amidase from *Nocardia farcinica* to improve the degradation of polyester PU pellets (Gamerith et al. 2016).

5. Analytical solutions for the measurement of polyurethane biodegradation

These last years, methods implemented for the measurement of PU biological degradation evolved. This chapter offers an overview of these different techniques. For the development of bioremediation or biological recycling processes, degradation of model molecules is generally the first step as it allows an easy identification of efficient degraders. These model molecules can be either low molar mass molecules or hydrophilic PU dispersion. Then, the degradation of more complex substrates, TPU and PU foams, is also addressed.

5.1. Model molecules

5.1.1. Low molar mass molecules

To cover the degradation of a maximum number of PU types, tracking the urethane bond hydrolysis appears as a relevant solution. In order to easily detect the hydrolysis of the urethane bond, low molar mass molecules can be designed. These urethane compounds are not soluble in water. Pre-dilution in organic solvents such as ethanol (Akutsu-Shigeno et al. 2006) or DMSO (Gamerith et al. 2016) is thus required.

Low molar mass N-tolylcarbamate molecules correspond to toluene with urethane linkage on one or two carbons of the aromatic ring bound to ethanol moieties (Owen et al. 1996). In Owen et al., aromatic amines resulting of the N-tolylcarbamate hydrolysis were extracted in chloroform and quantified by Gaz Chromatography coupled with Mass Spectrometry (GC/MS). This assay revealed that the degrading activity of the *Exophiala jeanselmei* strain REN-11A depends on the position of the urethane(s) around the aromatic ring. Toluene-2,4 and 2,6-dicarbamic acid diethyl ester were the most readily biodegradable molecules (Figure 1.8).

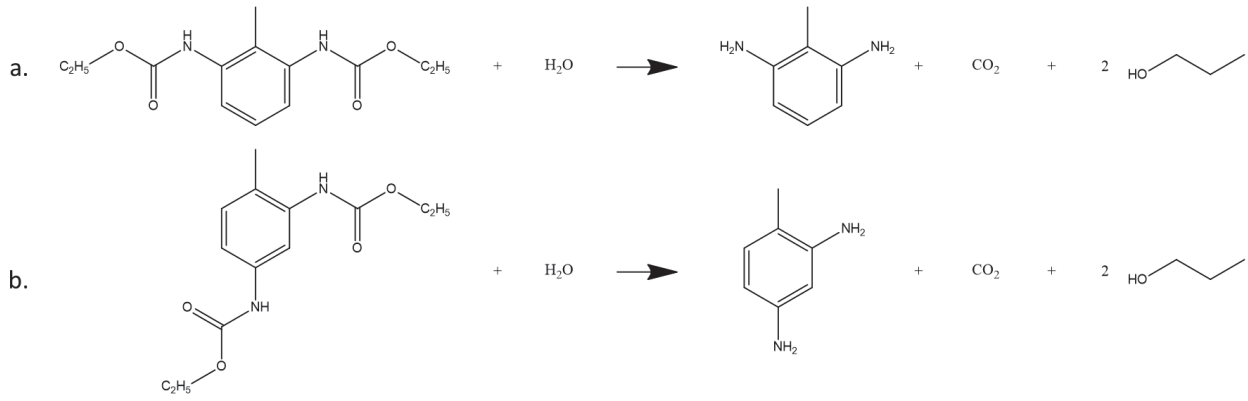


Figure 1.8 – Hydrolysis of (a) the toluene-2,6-dicarbamic acid diethyl ester into 2,6-toluene diamine and ethanol and (b) the toluene-2,4-dicarbamic acid diethyl ester into 2,4-toluene diamine and ethanol

Akutsu shinego et al., also proposed a set of molecules bearing a single urethane bond formed by reacting a di-isocyanate (2,4-TDI, 4,4'-MDI and HDI) with butanol (Akutsu-Shigeno et al. 2006). These compounds were degraded by *Rhodococcus equi* strain TB-60 and by a purified urethane-degrading enzyme secreted by this bacterium. Degradation products were extracted with ethyl

acetate and analyzed by GC/MS except for the HDI-based model molecules which degradation products were extracted with toluene under alkaline conditions. Because of the difficulties to detect aliphatic amines in GC/MS, amine coming from HDI-based molecule hydrolysis was derivatized using heptafluorobutyric acid anhydride following the method of Skarping et al. (Skarping, Dalene, and Mathiasson 1988).

To avoid the setup of complex analytical procedure such as GC/MS, Gamerith et al., proposed the synthesis of a model molecules based on 4-nitrophenol (Gamerith et al. 2016). Molecules based on this aromatic compound are well known as model substrates for enzymes such as esterase (4-nitrophenyl acetate) or amidase (4-nitroacetanilide). 1-methoxypropan-2-yl (4-nitrophenyl) carbamate was synthesized (Figure 1.9c). Its hydrolysis leads to the release of 4-nitroaniline that can be tracked and quantified by absorbance measurements at 405 nm.

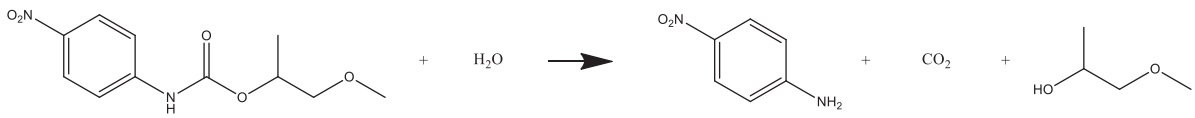


Figure 1.9 – Hydrolysis of the 1-methoxypropan-2-yl (4-nitrophenyl) carbamate leading to 4-nitroaniline and 1-methoxy-2-propanol (Gamerith et al. 2016)

The use of low molar mass urethane substrates is a good way to identify efficient degraders. However, the low steric hindrance of these molecules makes them far from being representative of actual PU materials which often present organized and crystalline structures and are much more hydrophobic. Activity assays on real and complex polymers must then be performed with these degraders.

5.1.2. Waterborne polyurethane dispersions

Thanks to the polymer particles nanometric size and homogeneity in water, waterborne polyurethane dispersions (WPUD) are particularly suitable to assess the degradation ability of enzymes and micro-organisms. Indeed, the specific surface is high thus maximizing interactions between the degrader and the polymer. Moreover, all the biodegradation reactions occur in aqueous media.

The most famous WPUD is the Impranil-DLN®, commercialized by Covestro (Germany) for textile coating applications. Particle size is estimated to range between 0.1 to 0.2 µm (Biffinger et al. 2015). This dispersion appears as a white, milky suspension containing 40% of polymer. The exact composition of the Impranil-DLN® is not fully known. A tentative structure has been proposed by Biffinger et al. based on poly hexane/neopentyl adipate polyester and HDI (Howard, Norton, and Burks 2012). Diethylene glycol is also a component of Impranil-DLN® (Gautam, Bassi, and Yanful 2007b).

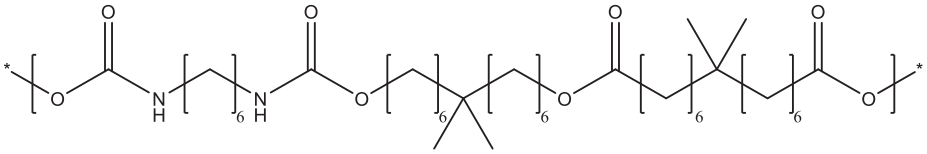


Figure 1.10 – Impranil tentative structure adapted from Biffinger et al. (Biffinger et al. 2015)

Impranil-DLN® has widely been studied because it has the particularity of becoming translucent when hydrolyzed as a result of water soluble molecules being released in the medium. Impranil-DLN® was used as "polyurethane biodegradation benchmark" for the first time in 1994 (Crabbe et al. 1994). A screening of soil fungi was performed on plate where Impranil-DLN® was mixed with an agar medium. Fungi were allowed to grow on it and a transparent halo appeared after few days if the micro-organism was producing degrading enzymes. Such agar plate technique has been then intensively used (Peng et al. 2014; Rowe and Howard 2002; Vega, Main, and Howard 1999; Howard, Crother, and Vicknair 2001) (Figure 1.11a & b). Impranil-DLN® is also suitable for assays in liquid media for both microorganisms (Russell et al. 2011; Alvarez-Barragan et al. 2016) (Figure 1.11c) and enzymes (Schmidt et al. 2017; Gautam, Bassi, and Yanful 2007b).

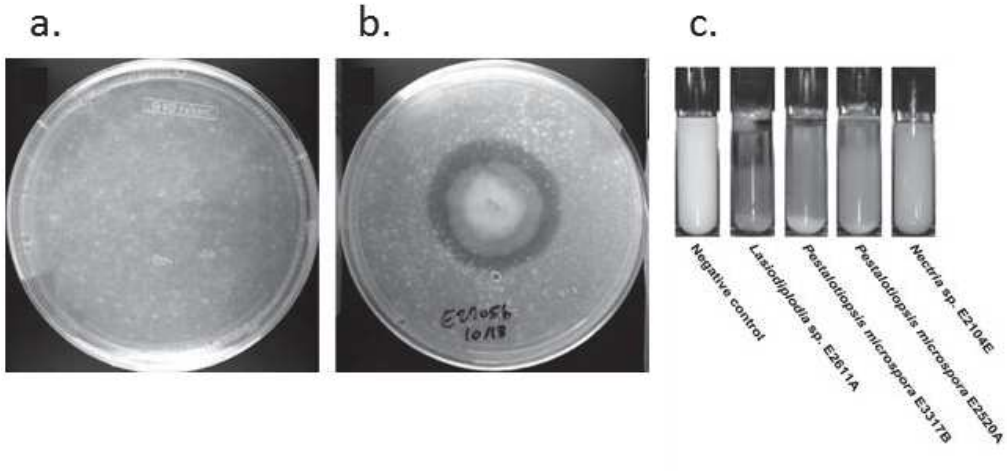


Figure 1.11 – Hydrolysis of Impranil-DLN®. Degradation assay in agar plate (a) negative control and (b) Pleosporales sp. strain E2705B after two weeks of incubation. (c) Assay in liquid media. Adapted from Russel et al. (Russell et al. 2011)

As the exact polymer structure is unknown, it is difficult to appraise the mechanism of degradation. Biffinger et al., used NMR and FT-IR to offer quantitative analysis of alcohol and carboxylic acid release after the ester bond cleavage (Biffinger et al. 2015). They also highlighted that polymer aggregation can occur when incubated with enzymes without any measurable degradation. Observation of the polymer is thus not sufficient to conclude on enzymatic degradation activity.

Ufarté et al., proposed to use Matrix Assisted Laser Desorption Ionisation - Time of Flight/Mass Spectrometry (MALDI-TOF/MS) to identify degradation by-products. Three peaks at m/z 682, 683 and 782 were specific of the bacterial degradation of Impranil-DLN®. All seemed to correspond to the formula $C_{36}H_{68}O_8N_2$, ionized with either Na^+ or I^-. The peak at m/z 683 could correspond to the isotope form of the molecule at m/z 682. However, no specific chemical structure has been suggested.

Alvarez-Barragan et al., presented the analysis of degradation by-products by GC/MS (Alvarez-Barragan et al. 2016). Almost none of the products identified correspond to the tentative structure described above (Figure 1.10) suggesting that the structure is much more complex than expected. The only corresponding molecule is the HDI. However, HDI cannot be a degradation

product as isocyanate are not stable in water. It can be either hexane diamine (HDA) or HDI-derivatives.

Despite some important limitations, Impranil-DLN® is thus a good model substrate to identify degraders that have great chance to be efficient afterwards on polyester PU. It provides a first approach that must then be confirmed. Hung et al. (2016) did not present Impranil-DLN® as a model but as a relevant coatings for military equipment which integrity must be preserved from microbial degradation (Hung et al. 2016). Esterase and lipase are mainly involved in the enzymatic degradation of Impranil-DLN®. But, up to now, there is no clear evidence that these enzymes, improperly called "Polyurethanase" (Ruiz et al. 1999; Stern and Howard 2000), effectively mhydrolyze the urethane bond.

Few other WPUD have been tested for degradation activities. Bayhydrol 110 (Covestro, Germany) is a polyester PU dispersion presenting the same properties of clarification than Impranil-DLN®. Translucent halo appeared when incubated with a strain of *Pseudomonas chlorographis* on an agar plate containing this WPUD (Howard, Crother, and Vicknair 2001). Poly Lack (Sayer Lack Mexicana, Mexico), a polyether PU was also tested on agar plate containing the polymer and minimal media (Alvarez-Barragan et al. 2016). It is possible to select strain able to grow on these but no clear-zone can be observed.

5.2. TPU and PU foams biodegradation

Both TPU and PU foams have been tested in biodegradation assays. These types of products display a wide and varied range of chemical structures. When the purpose is to evaluate the susceptibility to biological degradation of a newly synthesized material, then the polymer usually has a specific and known structure. In contrast, bioremediation and biological recycling studies on PU mainly involve commercial products of complex and often unknown chemical structure and composition.

As PU materials cannot be considered as "fully biodegradable" the biological degradation experiments described in the literature are always partial, leading to the recovery of a degraded polymer and, possibly, soluble degradation products released in the aqueous media (Figure 1.12). Techniques developed to evaluate the biological degradation of polymers can thus be oriented

towards the efficiency of the entire degradation system, the analysis of the degraded polymer or the identification and quantification of degradation products.

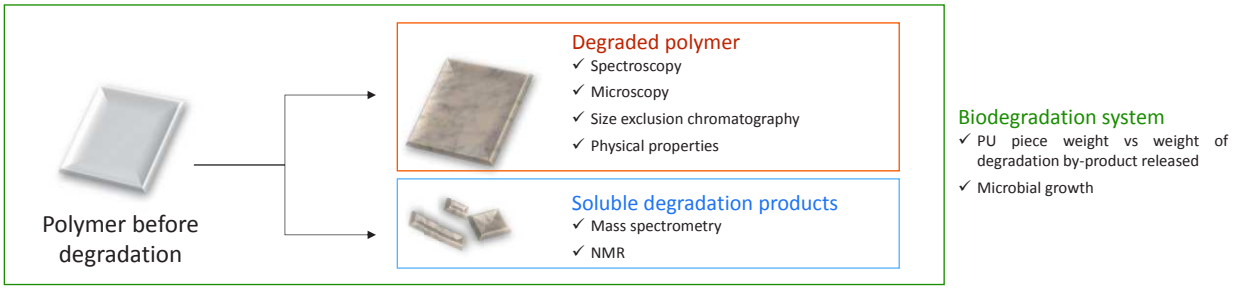


Figure 1.12 – Diversity of analytical methods for TPU and foam biodegradation measurement

Evaluation of PU foam degradation is more challenging than TPU one. Foams are highly complex systems with different components and additives. Side reactions during synthesis and foaming steps lead to the formation of isocyanurate and other uncontrolled bonds. Being insoluble in solvent, the potential of foams analysis is limited. As far as we know, only one publication deals with the enzymatic degradation of foams (Ng et al. 2017) with all other studies involving degradation by micro-organisms.

PU shape drives the specific surface and thus, the bioavailability which is of importance for the biological degradation. Thanks to their alveolar structure, bioavailability is greater for foams, especially for open-cell foams, as micro-organisms can easily circulate inside the material. Foams can also be ground to increase the bioavailability (Cregut et al. 2014). TPU for degradation assays can be used as thin film obtained by coating after solubilizing in an appropriate solvent, pouring on glassware and solvent evaporation (Woo, Mittelman, and Santerre 2000; Chen et al. 2016). Thin films obtained can reach a few dozen of micrometers (Phua et al. 1987). TPU films can also be obtained by thermoforming (Zhou and Xie 2017). TPU cubes (Nakajima-Kambe et al. 1995), pellets (Cosgrove et al. 2007) or sheets (Ibrahim 2009) are also used for PU degradation assays.

It is not recommended to sterilize TPU and foams by autoclaving for microbial experiments as most of the TPU would melt, become softer or start to degrade at autoclaving temperatures (120°C). A study comparing autoclaved and non-autoclaved poly(ether urea) PU material revealed

that no weight loss was observed after autoclaving. However, surface alteration appeared leading to bias in degradation measurement (Rafiemanzelat, Jafari, and Emtiazi 2015). Alternative such as rinsing with ethanol (Mathur and Prasad 2012; Cosgrove et al. 2010), UV exposure (Gogoi and Karak 2014) or both (Osman et al. 2018) are thus frequently employed.

5.2.1. Evaluation of the global biodegradation efficiency

The most widespread method to evaluate the global biodegradation is the weight loss of the PU. By weighing the polymer before and after degradation tests, the degraded polymer content and the amount of degradation products released in the media can be obtained. It is a straightforward and easy method to implement. Though, some bias can be noticed. If the degradation is too superficial and too low to lead to products release, the test will be considered as inefficient as no weight loss will be measured. Therefore, weight loss measurement must be associated with surface analysis of the polymer (Rafiemanzelat, Jafari, and Emtiazi 2015) in the case of low degradation extent or to analyze the early steps of the degradation. It is then necessary to remove all the biological materials that can remain on the polymer surface. Hard washing with ethanol is generally performed (Mathur and Prasad 2012; Urgun-Demirtas, Singh, and Pagilla 2007). Non-ionic surfactant such as TritonX-100 (Polyethylene glycol tert-octylphenyl ether) (1% v/v) have also been suggested to remove reversibly bounded enzymes and cells (Ciardelli et al. 2004). This cleaning step is particularly tricky for the fungal degradation of foams as filaments are deeply trapped into the bulk structure of the samples. It was recently suggested to use 0.88% (wt/vol) sodium hypochlorite for 18 h to destroy and remove the remaining mycelium (Alvarez-Barragan et al. 2016).

When enzymatic degradations are performed, weight loss kinetics are generally set up (Dogan et al. 2017; Zhou and Xie 2017). Enzyme denaturation over time correspond to a loss of the enzyme 3D structure and thus to a loss of enzymatic activity. To cope with this phenomenon, enzymatic solutions are frequently renewed (Phua et al. 1987). In-between the removal and addition of enzymes, polymer pieces are washed, dried and weighed.

As a parameter of global assay efficiency, evaluation of the ability of a micro-organism to grow on PU, when the polymer is used as sole source of carbon (or carbon and nitrogen), is a common

practice (Cooney 1969). Indeed, microbial development means that micro-organisms are able to depolymerize the PU and metabolize degradation products for growth.

It is possible to quantitatively follow the bacterial growth through the MacFarland method which estimates the number of bacteria thanks to turbidity measurement at 600 nm (Bayan and Karak 2017; Fernandes et al. 2016; Gogoi and Karak 2014). Colony forming unit (CFU) is another way to count bacteria (Urgun-Demirtas, Singh, and Pagilla 2007; Crookes-Goodson et al. 2013). Briefly, bacteria incubated with polymers are sampled, diluted and poured on an agar plate containing a rich medium. Colonies are counted after an overnight incubation. By contrast, the evaluation of fungal growth is only qualitative.

Mineralization of polymers by micro-organisms in aerobic conditions leads to the production of CO_2 with O_2 consumption. Online sensors allow measuring both evolutions. These variations must be compared to a negative control and without polymers (Cregut et al. 2014) or with an inert polymer such as low density polyethylene (Rattanapan et al. 2016) and to a conventional biodegradable positive control such as cellulose (Gómez et al. 2014) or sodium benzoate (Rattanapan et al. 2016) incubated with the same inoculum. For instance, low O_2 consumption and CO_2 release were measured during 28 days of degradation of a ground polyether PU foam revealing low degradation by an acclimated microbial consortium (Cregut et al. 2014).

CO_2 release during PU mineralization is associated with pressure increase. The pressure can be measured by a Sturm test (Standard OECD 301 B) (Shah et al. 2016). This test is used for readily biodegradable materials and usually lasts 28 days. Rattanapan et al., used this assay to measure the biodegradation of a biobased polyester PU foam with a long time of incubation (60 days) (Rattanapan et al. 2016). Indeed, after 30 days, 7 to 11% degradation was observed while a higher degradation rate occurred during the 30 last days leading to a maximal degradation yield of 46 wt%.

Under anaerobic digestion, CH_4 is produced proportionally to the polymer consumption. Gomez et al., proposed to compare the biological susceptibility of polyether PU foams under composting, soil burial and anaerobic digestion according to three ASTM standard methods based on CO_2 or CH_4 measurement (Gómez et al. 2014). These methods are ASTM D5988-03 (Standard Test

Method for Determining Aerobic Biodegradation in Soil of Plastic Materials or Residual Plastic Materials After Composting) (International 2003b), the ASTM D5338-98 (Standard Test Method for Determining Aerobic Biodegradation of Plastic Materials Under Controlled Composting Conditions) (International 2003a) and the ASTM D5511-02 (Standard test method for determining anaerobic biodegradation of plastic materials under high-solids anaerobic-digestion conditions) (International 2002). The best degradation was observed for a bio-based PU foam after 320 days of soil burial.

Methods involving CO_2, O_2 or CH_4 measurement present a low throughput. For instance, flasks recommended for OECD 301 series range from 2 to 5 liters. These methods are mainly oriented towards the evaluation of the biological sensitivity of newly synthesized or commercial PU, especially foams.

5.2.2. Study of the degraded polymers

The first assessment of polymer degradation is eye-naked observation, sometimes sufficient to evaluate the damages. Change in color, roughness or shape can be noticed (Pilch-Pitera 2012). These observations can be completed by surface analysis such as spectroscopy and/or microscopy, polymer molar mass evolution by SEC or modifications of physical properties.

Spectroscopy techniques

FT-IR (Fourier-transform infrared spectroscopy) relies on the fact that most molecules absorb in the infra-red region. This absorption corresponds specifically to the bonds present in the analyzed molecules, and their movements. Absorption spectra thus provide information on the chemical structure of the polymer as a finger-print. FT-IR is particularly popular for PU degradation analysis because of its accuracy and rapidity and because FT-IR is a nondestructive method. The sample is recovered without damage after the analysis. By reflection (Attenuated Total Reflectance or ATR), surface analysis can be easily performed to measure superficial biological degradation. Several spectral zones have been identified for PU, particularly regarding PU degradation (Table 1.3).

Table 1.3 – Absorption band assignments of PU FT-IR Spectroscopy adapted from Sponton et al., 2013 (Spontón et al. 2013)

Bands	Wavelength (cm^{-1})	Assignment
N-H O-H	3400	N-H hydrogen bon and Oh stretching vibration band
-CH2	2990	Methylene stretching vibration asymmetric modes
-CH	2880	Methylene stretching vibration symmetric modes
C=O	1630	Urea hydrogen bond stretching vibration
	1660	Free urea stretching vibration
	1700	Urethane hydrogen bonded stretching vibration
	1720	Free urethane stretching vibration
	1750-1725	Ester and amide stretching vibration
-C=O-NH-	1540	N-H bond vibration (in secondary amide)
-CH2	1470-1430	Methylene groups stretching vibration asymmetric modes
C-N	1290-1220	C-N stretching vibration modes
C-O-C	1190	Ether stretching vibration modes

Different issues can be addressed on the analysis and interpretation of the spectra. For instance, partial similarities between bonds e.g., urethane and ester groups complicate the spectra interpretation for polyester PU. Moreover, both hydroxyl (OH) moieties, resulting from ester and urethane degradation, and amine (NH) moieties, resulting of urethane degradation, absorb around 3400 cm^{-1} (Figure 1.13a). For polyester PU, the increase of this large band is generally attributed to ester- or to both ester and urethane hydrolysis (Spontón et al. 2013; Oprea 2010), but have already been interpreted exclusively as the cleavage of the urethane bond (Umare and Chandure 2008) .

Similarly, it is difficult to interpret changes in spectra linked to the carbonyl bond (C=O) that appear in ester (1750-1725 cm^{-1}), urethane (1700 cm^{-1}) and urea bonds (1630 cm^{-1}). In polyester PU, there is often a unique broad signal representing both urethane and ester carbonyl bond. Its decrease is generally attributed to ester bond hydrolysis (Shah et al. 2016; Schmidt et al. 2017) but sometimes, attributed only to urethane bond hydrolysis in polyester PU (Ozsagiroglu, Iyisan, and Guvenilir 2012; Gómez et al. 2014) (Figure 1.13). Interpretation of the peak intensity increase

at 1530 cm^{-1} is linked to urethane bond hydrolysis. Oprea *et al.* suggested that an increase of this signal is related to urethane bond hydrolysis (Oprea 2010) while others suggested that a decrease of this signal attests for urethane bond degradation (Oceguera-Cervantes et al. 2007; Sarkar and Lopina 2007). Concluding on the variation of this signal upon degradation appeared thus tricky.

Other peaks are sometimes considered for PU degradation. For instance, emergence of a peak at 2250 cm^{-1} after degradation has been attributed to isocyanate (NCO) release (Shah et al. 2016) (Figure 1.13). However, the high reactivity of the isocyanate groups makes this interpretation highly confusing. This signal could correspond to atmospheric CO_2 (Gerakines et al. 1994).

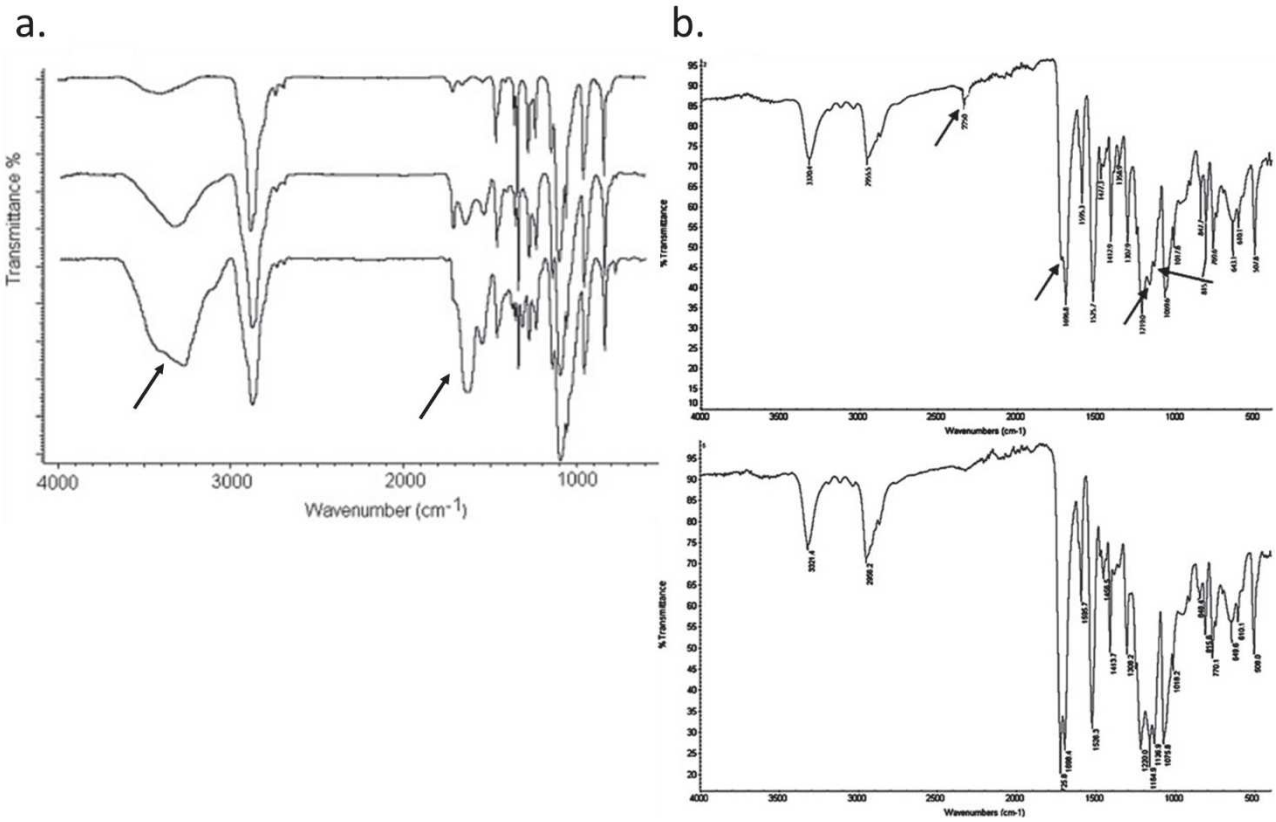


Figure 1.13 – FT-IR spectra (a) of a poly(ester ether) urethane before (top), after 90 days (middle) and after 130 days (bottom) of fungal degradation, adapted from Oprea et al., 2010 and (b) of a polyester PU incubated 30 days with (bottom) and without (top) bacteria adapted from Shah et al., 2016

Since high variation on the interpretations from FTIR spectra are observed through the literature, especially concerning polyester PU, other methods should be used to confirm urethane bond cleavage for PU degradation.

Kay *et al.*, suggested to consider the decrease of the ratio ester (C=O) /ether (1720 cm^{-1}/1125 cm^{-1}), the ratio urethane (NH)/ether (1630 cm^{-1}/1125cm^{-1}) and the ratio aryl (C=C)/ether in order to provide a semi quantitative analysis of the degradation of a polyether PU (Kay, McCabe, and Morton 1993). The ratio ester/ether decrease after degradation with a strain of *Corynebacterium* while the ratio urethane/ether and aryl/ether remain stable meaning that the ester bonds are affected by the bacterial degradation. Zhang et al., took the band at 1463 cm^{-1} corresponding to CH_2 moieties to normalize the results (Zhang et al. 1994).

Recently, Raman spectroscopy has been applied to monitor the biodegradation of a polyether polyurethane foam which is among the most recalcitrant PU (Cregut et al. 2013). This technique allowed concluding on the amorphous region degradation of the foam by a microbial consortium while crystalline region remained unaffected.

Infrared spectroscopy has recently been combined with atomic force microscopy (AFM-IR) (Biffinger et al. 2014; Barlow et al. 2016). It helped understanding interaction between the material, a polyether TPU of 200 nm thickness and a micro-organism, *Pseudomonas protegens* Pf-5, at nanoscale.

Microscopy

For the assessment of morphological surface modification of PU, microscopy, particularly scanning electron microscopy (SEM) is employed. SEM allows a qualitative evaluation of the degradation on the surface after treatment.

Cracks or holes are observed on the surface of degraded TPU. Enzymatic degradation generally leads to cracks (Figure 1.14a) or holes (Figure 1.14b) homogeneously spread at the polymer surface (Schmidt et al. 2017; Ozsagiroglu, Iyisan, and Guvenilir 2012) while degradation with a microbial consortium leads to irregularities (Figure 1.14c) (Zafar, Houlden, and Robson 2013; Das et al. 2017; Thirunavukarasu et al. 2015). For instance, Das et al., showed the appearance of cracks at the surface of a polyester TPU degraded under composting conditions. Depth of the cracks, corresponding to fungal mycelium development, increased to finally let appear holes (Das et al. 2017).

SEM observations of the fungal mycelium propagation inside a PU foam highlights the higher biodegradability of open-cell foam compared to closed-cell foam. The strut of cells appeared distended, leading to collapse of the alveolar structure (Figure 1.14d) (Alvarez-Barragan et al. 2016). Degradation is efficient in a PDB medium (rich medium) but it is specified that no degradation was observed by weight loss nor microscopy in a minimal media. Small holes appeared when incubated with one of the three tested strains confirming enzymatic action. Holes in the walls and struts of the foams structure were already described previously (Figure 1.14e) (Gautam et al. 2007).

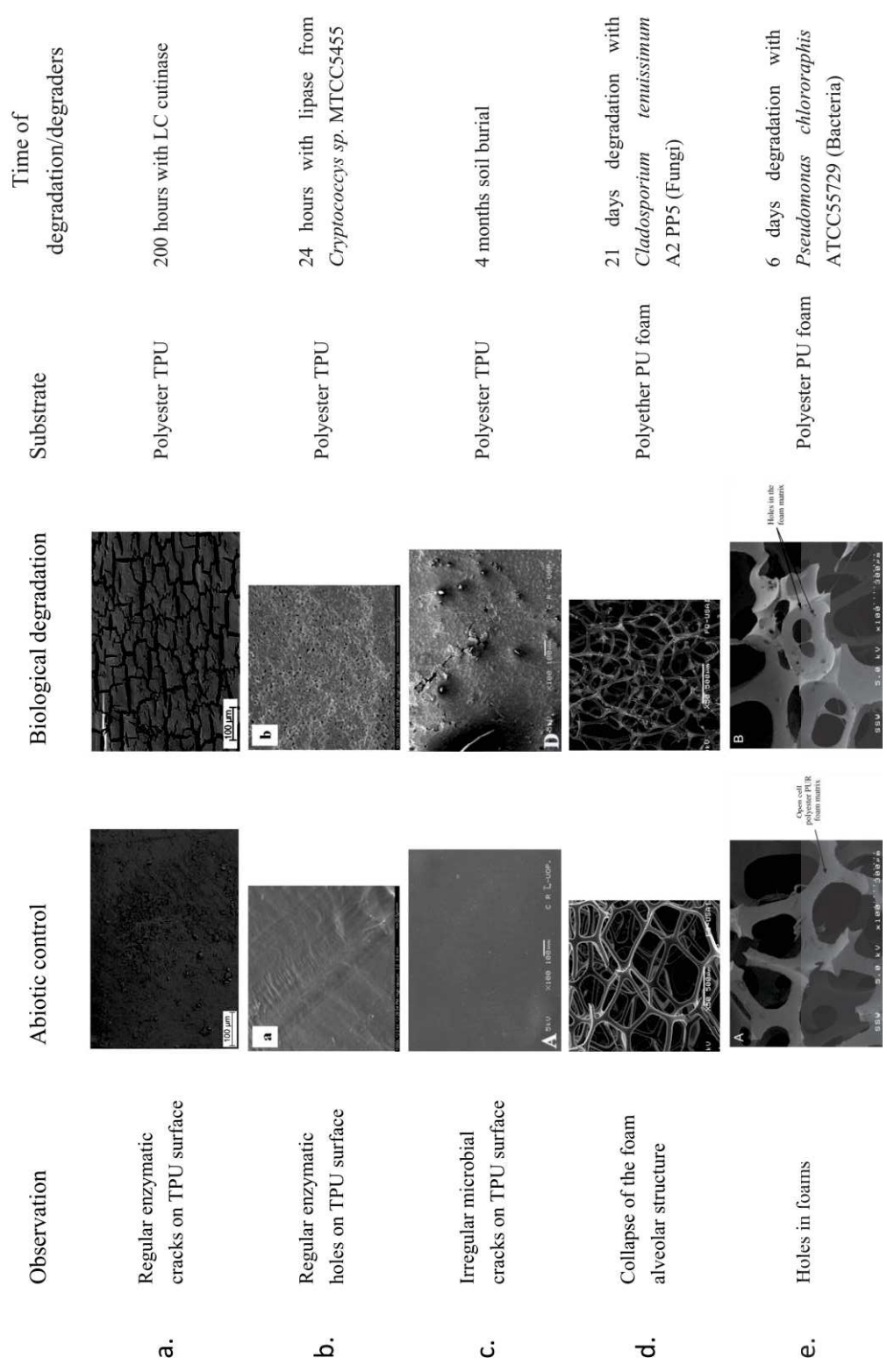


Figure 1.14 – SEM observation of PU biological degradation adapted from (a) Schmidt et al., 2017, (b) Thirunavukarasu et al., 2015, (c) Khan et al.,2017 , (d) Alvarez-Barragan et al., 2016 and (e) Gautam et al., 2007

SEM is also used to evaluate microbial growth. For instance, *Micrococcus* biofilm formation on the surface of a poly(ether urea) PU was shown by microscopy (Rafiemanzelat, Fathollahi Zonouz, and Emtiazi 2013). SEM can cope with the limitation of available assays to evaluate fungal growth on carbon depleted media containing polymer. Huang et al., highlighted the fungal growth on polymer surface using microscopy (Huang et al. 2016).

Size Exclusion Chromatography (SEC)

SEC analysis allows the determination of molar mass distribution of polymers. This measurement relies on the separation of the polymer chains in a column according to their length. . For that, the polymer is solubilized in an organic solvent as a mobile phase such as THF, chloroform, dimethylformamide (DMF). Then, the solution runs through a fix column packed with porous beads with different sizes pores. Short chains pass through the pores while longer chains cannot enter the pores and are eluted more rapidly. Detection can be done with e.g., UV diode array detector or refractive index detector (RID). UV, generally at 254 nm is adapted for PU containing aromatic rings such as 4,4'-MDI- or TDI-based PU. Since the polymer has to be soluble in an organic solvent this method is only suitable for TPU analysis.

Analysis and comparisons of chromatograms can be performed to analyze on PU degradation (Christenson et al. 2006; Rafiemanzelat, Fathollahi Zonouz, and Emtiazi 2013). Molar masses are usually determined with polystyrene standards. Because of its aromatic ring this standard is adapted to both UV and RI detection. Poly(methyl methacrylate) (PMMA) standards can also be used but this is only suitable for RI. Three main parameters are usually considered: the number average molar mass (Mn), the weight average molar mass (Mw) and the dispersity (Đ) which is the ratio of Mw over Mn.

Polymer degradation leads to changes in the molar mass distribution. The most common observed variation on PU biodegradation studies is a decrease of the Mw whereas Mn remains unchanged, leading to a decreasing Đ values (Schmidt et al. 2017). Mw being more sensitive to long polymer chains contribution this is consistent with the cleavage of the long chains into lower molar mass molecules. In Ferris et al., only the Mw was found to decrease (Ferris et al. 2010). Changes in molar mass distribution reveal global degradation in the bulk material and not only

what is occurring at the surface of the polymer (Shah, Hasan, et al. 2013; Shah, Krumholz, et al. 2013).

Rafiemanzelat et al. described a bi-modal SEC profile with a high and a low molar mass distribution after 4 months of a poly(ether urea) PU soil degradation (Rafiemanzelat, Fathollahi Zonouz, and Emtiazi 2013) resulting from the cleavage of the long polymer chains into shorter ones (Figure 1.15).

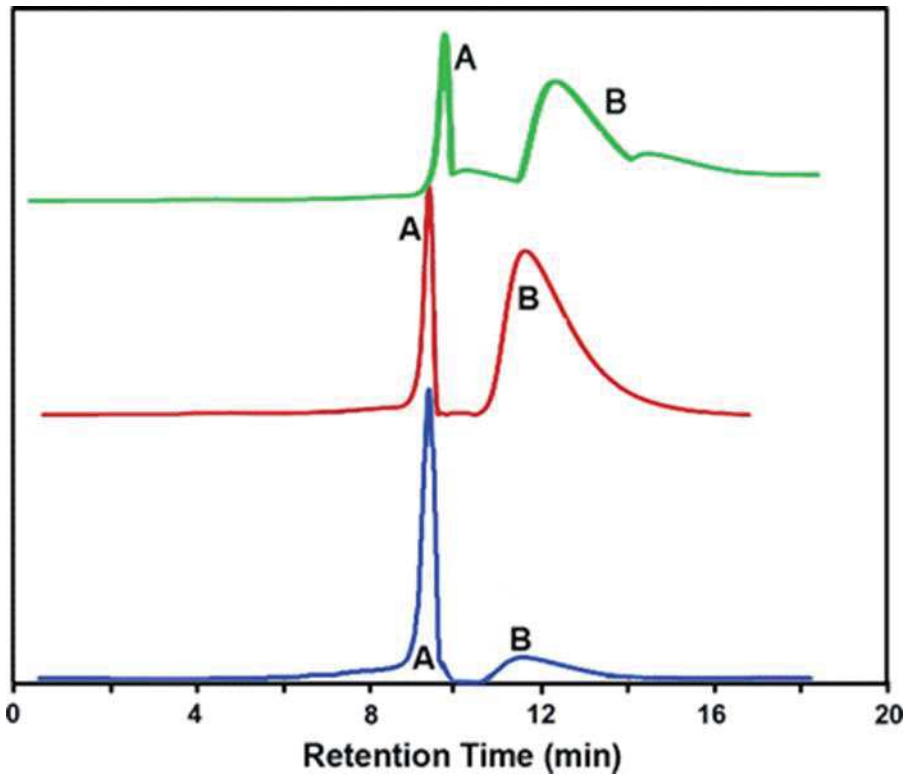


Figure 1.15 – SEC chromatograms of a poly(ether urea) PU before (bottom), after 4 months (middle) and after 6 months (top) of soil burial, adapted from Rafiemanzelat et al., 2013

Throughput of SEC analysis is rather low since a run generally lasts about 40 minutes. Lots of SEC analyses without repetitions are presented in the literature (Shah, Krumholz, et al. 2013; Rafiemanzelat, Fathollahi Zonouz, and Emtiazi 2013; Ferris et al. 2010; Brzeska et al. 2015; Ratner, Gladhill, and Horbett 1988). However, standard deviations are absolutely necessary to appraise the significance of change in Mw, Mn or Đ. Only few publications on PU biodegradation offered

robust SEC analysis with several repetitions of the analysis (Schmidt et al. 2017; Kuang and Mather 2018).

Modifications of the physical and physico-chemical properties of PU degraded material

Observation of modifications in the physical and physico-chemical properties of a polymer can be an indirect method of assessing the biodegradation since the polymer chains cleavage will often affect some of these properties.

Loss of mechanical properties such as tensile strength are generally observed after significant biological degradation of a material. The tensile strength evaluates the material resistance to traction. Tensile test measurement allows, for example, to evaluate material elasticity and the behavior at break (Phua et al. 1987). For instance, after 24 months of composting, a decrease of the tensile strength from 20 to 10 MPa was measured for a poly(ether urea) TPU. In similar degradation conditions, samples of polyester PU were already broken (Krasowska et al. 2012). This technique is also adapted to flexible PU foam. The tensile strength increased and the elongation at break decreased after 60 days of incubation with a strain of *Pseudomonas* for polyester PU foams (Spontón et al. 2013) (Figure 1.16, PU-1 and PU-2). No change of mechanical properties was observed for the polyether PU foam before and after degradation (Figure 1.16 PU-3).

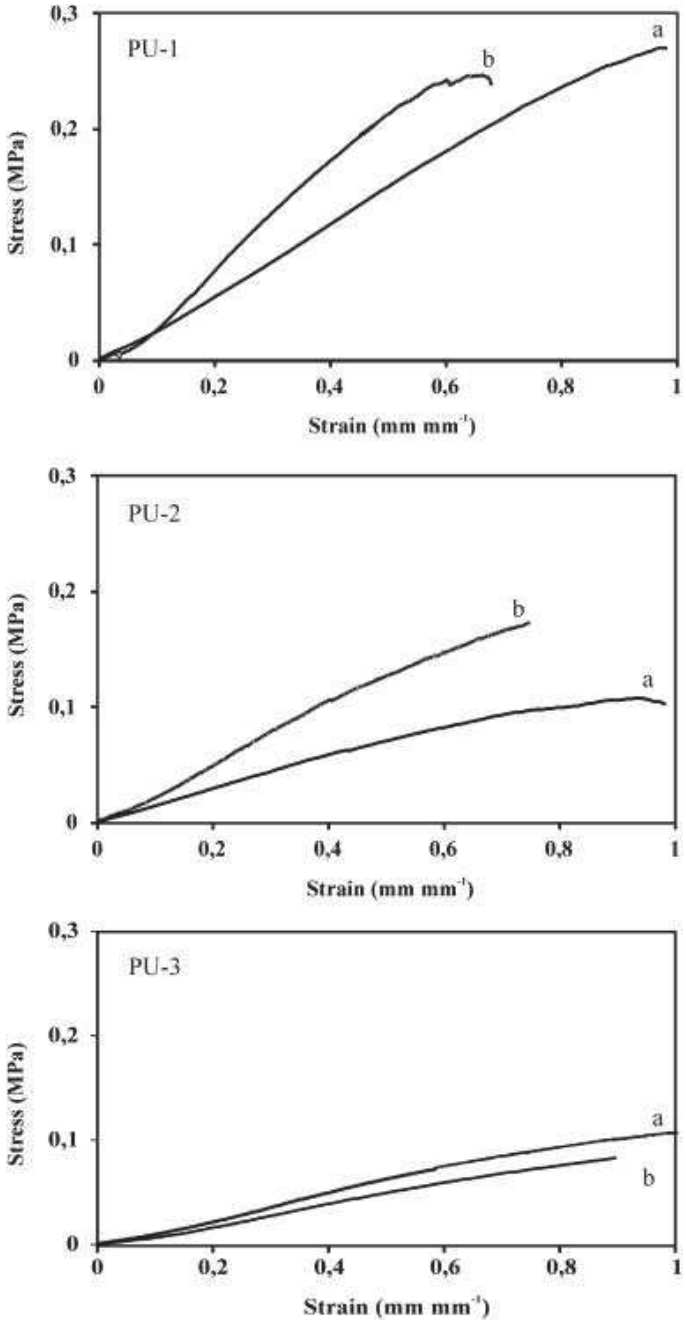


Figure 1.16 – Mechanical properties of the foams (a) before and (b) after 60 days of biodegradation by Pseudomonas sp. (PU-1) polyester PU foam with 25% of castor oil, (PU-2) a polyester PU foam with 75% and (PU-3) a polyether based PU foam, adapted from Sponton et al., 2013

PU thermal stability may also be affected by biological degradation. This property can be measured by thermogravimetric analysis (TGA) where the weight evolution of sample is recorded while the temperature is increased in a furnace, under air (oxidative) or N_2 (non-oxidative)

environment. For most polymers the analysis temperature ranges from 0 to 600-800°C. Zones of weight loss correspond to specific structure degradation and/or distinct mechanisms. The starting and maximum thermal degradation temperatures are usually determined to compare thermal stability between samples. For polyester TPU, a first zone from 100 to 300°C corresponds to the release of volatile compounds such as additives. Although urethane bonds present a reversibility at around 200°C (Delebecq et al. 2013), urethane bonds degradation induces a weight loss between 300 and 400°C while ester bonds cleavage results in a weight loss between 400 and 500°C (Cangemi et al. 2006; Mathur and Prasad 2012). A decrease in the weight loss occurring between 400 and 500°C was observed after biological degradation (Mathur and Prasad 2012) (Figure 1.17). It revealed a decrease of the ester bonds content in the polymer and thus, the biological hydrolysis of these linkages. Beyond the type of linkage affected, TGA may provide information on the material part affected. For instance, a poly(ether urea) PU was incubated for one month with a strain of *Bacillus.* The observed changes in the material thermal stability attested for a higher proportion of hard segments and thus, a degradation occurring at the soft segment domains containing ether bonds (Rafiemanzelat, Jafari, and Emtiazi 2015).

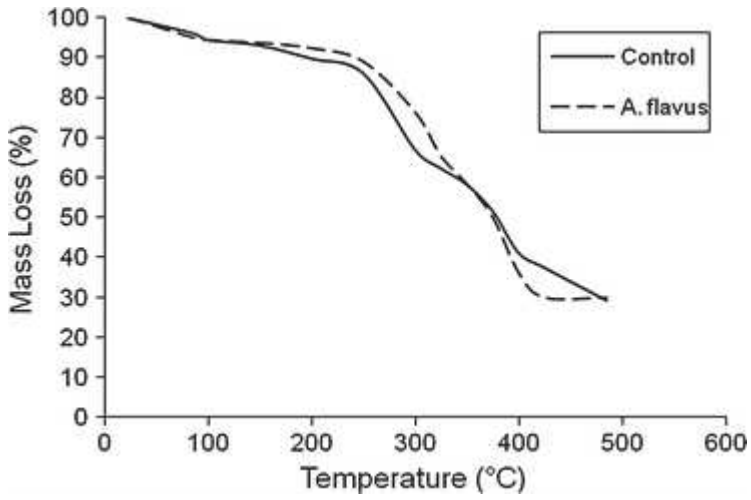


Figure 1.17 – Thermogravimetric curve for polyurethane after 30 days of incubation, with and without (control) A. flavus (ITCC no. 6051), adapted from Mathur et al., (Mathur and Prasad 2012)

TGA is also adapted to cross-linked foams. In his study, Gomez et al., compare the composting of a petroleum based and a biobased foam (Gómez et al. 2014). TGA was combined with MS analysis

to identify the gaseous products of thermal degradation. For instance, fatty acid methyl esters of the biobased polyester PU foam were thermally degraded between 110 and 200°C. A decrease of the thermal degradation on this zone after 50 days of composting revealed the degradation of the fatty acid methyl esters part of the PU. Urethane bond of the petroleum-based polyether PU foam were found to be degraded between 240 and 400°C but no difference was observed before and after composting.

Biological degradation can modify the hydrophobicity of a PU material. Indeed, disruption (cracks, holes…) observed on the material surface offer higher surface area of access for water and thus higher hydrophilicity. Consequently, the higher is this contact angle, the more hydrophobic is the material. To evaluate the hydrophobic/hydrophilic balance, a drop of determined liquid such as water is deposited on the polymer surface and the contact angle is measured. A shift from 90° to 63° have been measured after 320 days of soil burial of a polyester PU (Aranguren, González, and Mosiewicki 2012).

5.2.3. Analysis of the degradation products

The soluble degradation products are indicators of degradation efficiency and mechanism. The chemical structures of most frequently identified degradation products after PU biological degradation are shown in Figure 1.18.

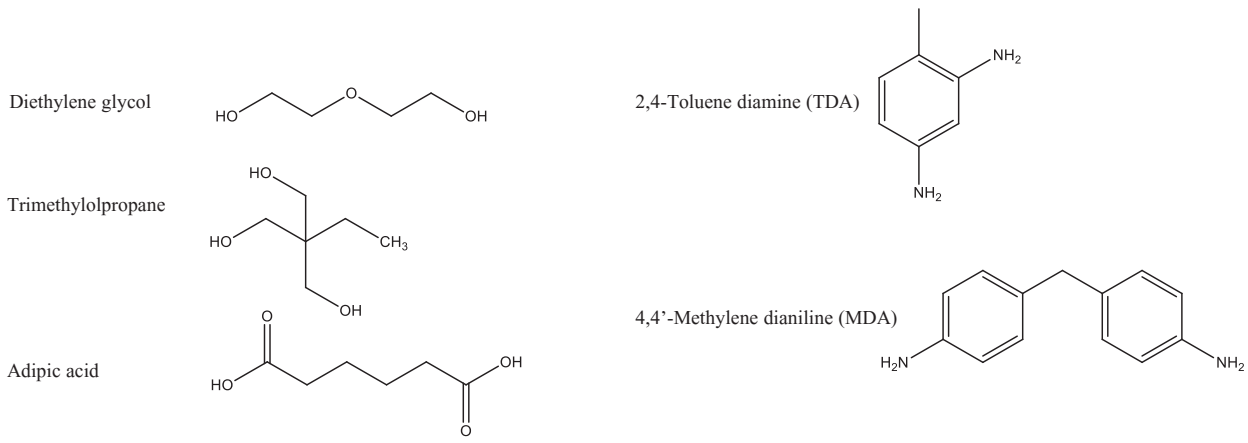


Figure 1.18 – Degradation products frequently identified after PU biological degradation

Quantification of these products is possible with the measurement of the Total Organic Carbon (TOC) of the soluble fraction (Yamamoto et al. 2007). This measurement can be performed only for enzymatic reactions as the amount of carbon from the enzyme remains stable in time contrary to microbial cultures which involve growth and thus an increase in carbon content. Yamamoto et al., used this method to evaluate the degradation of several PU based on lysine diisocyanate (LDI) with various proteases. For instance, 518 ppm of carbon from degradation products were released after the degradation of a PU based on LDI and ethylene glycol, representing 44% of the theoretical carbon of the polymer. Another way to measure the release of soluble degradation products is the use of radiolabeled polymers. They were synthesized with ^{14}C molecules such as ^{14}C-TDI, ^{14}C ethylene diamine or ^{14}C 1,4-butanediol or ^{14}C 1,6-HDI (Santerre et al. 1994; Woo, Mittelman, and Santerre 2000). Increase of radiolabeled-based molecules in the supernatant are quantified in counts per minutes (CPM). However, the cost and the hazardous exposure to radioactivity for the experimenter limit its use. Both TOC and radioactivity measurements allow precise quantification of the degradation products but these techniques do not give information about the chemical structure of these degradation products.

Another indirect way to assess PU biodegradation is to monitor properties that could be impacted by the degradation products. For instance, some of the molecules released after biological degradation might be toxic. This is the case of 4,4'-methylene dianiline (MDA) resulting for the hydrolysis of MDI-based PU which is known to be carcinogenic (McQueen and Williams 1990). The toxicity of the soluble fraction containing degradation products can be evaluated using Microtox® bio-assay. This assay relies on a marine bacteria, *Photobacterium phosphoreum* (Spontón et al. 2013). A decrease of the IC_{50}, concentration for which the inhibition is of 50%, was observed for liquid medium of a polyester PU foam incubated 60 days with *Pseudomonas sp* attesting for the presence of MDA as degradation product and thus the effective degradation of such PU material.

Mass spectrometry preceded by liquid (Elliott et al. 2002) or gas chromatography (Pérez-Lara et al. 2016) are methods of choice to identify the degradation products. The detection of specific amines resulting of the cleavage of urethane bonds finally appears as the best way to confirm the hydrolysis of this bond. It is interesting to notice that the degradation with the cholesterol

esterase of a PU based on TDI and PCL leads to the release of low molar mass urethane molecules based on TDI but no TDA was detected (Wang, Labow, and Santerre 1997) (Figure 1.18). The urethane bond has not been cleaved. MDA was the unique aromatic amine released from polycarbonate-based PU synthesized with diverse diisocyanates (HDI, HMDI and MDI) after hydrolysis with the cholesterol esterase (Tang, Labow, and Santerre 2003b). Gamerith et al., used liquid chromatography/electrospray/time-of-flight mass spectrometry (LC/ESI/TOF-MS) and also detected MDA and MDA derivatives after the hydrolysis of a polyester PU incubated with a *Nocardia farcinica* polyamidase. It can be noticed that none of these publications presents MDA quantification. Moreover, the degraded polymer was not analyzed after the degradation assays. Therefore, the extent of the biological degradation cannot be appraised.

Identification of degradation products is the best route to understand PU degradation mechanisms. For this purpose, an enzymatic degradation is the favorite route. Indeed, for microbial degradation, the results could be biased by the possible assimilation of some molecules by the micro-organisms. Thus, the recovered degradation products are those that cannot be assimilated by the micro-organisms.

Efficient recovery of the degradation products for their analysis is another issue. Indeed, the liquid fraction of a degradation assay is a mixture containing salts, enzymes, eventually microbial cellular debris and degradation products that are molecules released from the polymer. Several strategies were suggested to recover only the degradation products from PU degradation assays. It can be performed by solvent extraction using, for example, ethyl acetate (Shah et al. 2016), acetonitrile (Tang, Labow, and Santerre 2003b) or ethyl ether (Spontón et al. 2013). Instead of solvent extraction, it is possible to remove enzymes by filtration (Wang, Labow, and Santerre 1997). Gamerith et al., added one volume of methanol and acidified the supernatant to pH 3.5 so the proteins precipitate and can be removed by centrifugation (Gamerith et al. 2016)..

Besides giving information on the degradation mechanism, released molecules resulting from PU biological depolymerization can also be considered as valuable products and used as building blocks for second generation polymer synthesis. In the review of Cregut et al. 2014, economic value of major building blocks was evaluated showing the interest of the recovery of molecules

such as diethylene glycol, adipic acid or trimethylol propane which are products often identified after PU biological degradation (Figure 1.18).

5.3. Conclusion

PU are versatile polymers with high variability of the structure, chemical composition, formulation, morphology, shape, with a direct impact on the biodegradation mechanism and kinetics. From the published literature, a large variety of potential or efficient degraders can been identified such as fungi, bacteria or enzymes. Because of the diversity of substrates and analytical tools, direct comparison of these degraders does not appear as an easy task.

To tackle the degradation of the widest range of PU, the urethane bond cleavage appears as the key parameter. However, only few techniques provide undeniable proof of the urethane bond degradation. Development of new techniques such as Raman spectroscopy or LC/ESI/TOF-MS helps going forward in the resolution of this issue. Microbial degradation of PU still remains a black box. Analysis of the set of enzymes produced by degrading-micro-organisms is lacking to understand the mechanisms involved in PU degradation.

Biological recycling of PU is a great challenge to address in the coming years. Efficient controlled PU biodegradation is the premise of this development. Up to now, studies on PU biodegradation only focused on simple PU model far from being representative of real and complex commercial PU formulations (products containing additives). Applicability on mainstream PU waste still need to be attested. As far as we know, only one study deals with the biodegradation of a real PU waste. Gautam *et al.,* described the successful degradation of a waste polyester PU foam with a strain of *Pseudomonas chlororaphis* (Gautam et al. 2007). Attempt to fit with real PU waste has recently been performed by Alvarez-Barragan *et al.* by studying the degradation of polyether-PU foam synthesized with and without the addition of a fire retardant tris(1,3-dichloro-2-propyl)phosphate (TDCPP) (Alvarez-Barragan et al. 2016). The TDCPP-containing foam was found to be less sensitive to biodegradation thus highlighting the need for considering thoroughly the presence of additives in PU biological degradation assessment.

Polluting waste management such as landfilling or incineration will no longer be suitable solutions. Limitation of the pollution linked to PU waste is an outcome deeply expected from recycling. Efficient biological recycling path for PU will support economical value to its waste.

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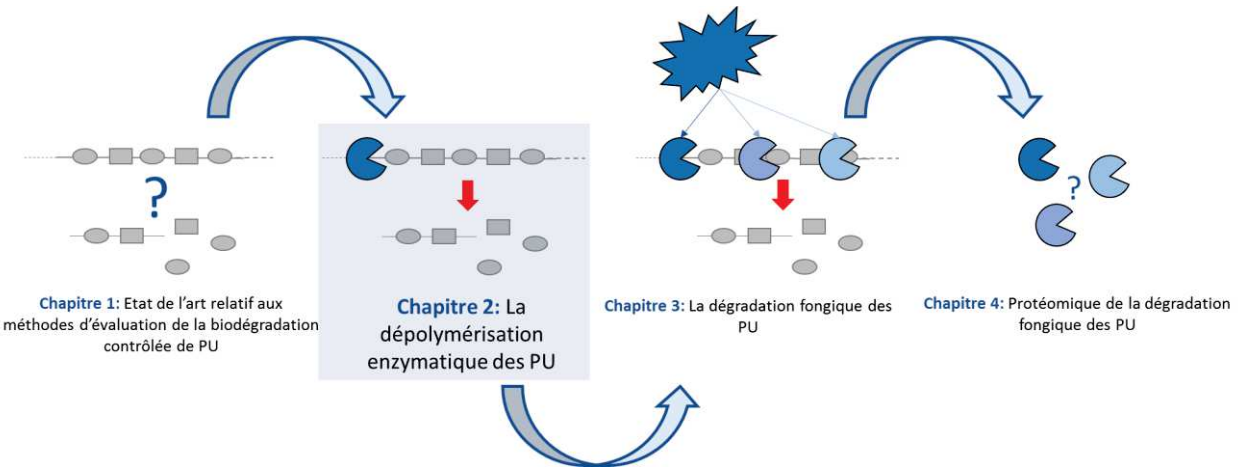
Conclusion

Ce chapitre bibliographique propose une vue d'ensemble des différents aspects relatifs à la dégradation biologique des PU. Tout d'abord, les diverses structures des PU ainsi que les principales voies de gestion des déchets émanant de ces matériaux ont été décrites, révélant la complexité de ce sujet. Les différents acteurs de la biodégradation: consortia microbiens, souches de micro-organismes ou enzymes, ainsi que leurs particularités ont ensuite été présentés et discutés. Finalement, le cœur de ce chapitre repose sur l'étude de la biodégradation des PU, présentée principalement sous l'angle de vue des méthodes analytiques permettant l'évaluation voire la quantification de cette dégradation. Un large panel de méthodes d'analyses a ainsi été présenté et discuté.

En effet, pour la partie expérimentale de cette thèse, la réalisation de criblages enzymatiques et microbiens est nécessaire afin de sélectionner les entités biologiques d'intérêts. Parmi les méthodes d'analyses décrites dans ce chapitre, certaines peuvent être adaptées à un débit élevé et donc, convenir au développement de criblage. Au regard de la littérature, l'utilisation de molécules modèles apparait, entre autres, comme un point de départ intéressant pour l'étude de la dégradation biologique des PU. Cet état de l'art montre également que pour pouvoir proposer une analyse fine et approfondie de la dégradation biologique des PU, il conviendra d'évaluer l'efficacité globale des systèmes de dégradation mais également d'analyser les polymères dégradés et les produits de dégradation.

Chapitre 2. La dépolymérisation enzymatique des polyuréthanes



Introduction

L'objectif des travaux décrits dans ce chapitre est donc d'identifier et de caractériser des enzymes capables de dépolymériser les polyuréthanes. Pour cela, une collection de 50 enzymes a été mise à disposition par Protéus (Nîmes, France). L'entreprise Soprema (Strasbourg, France) a synthétisé et fourni les différents substrats à base de polyuréthanes, à notre demande.

Le développement de criblages permettant de sélectionner les enzymes d'intérêt parmi les 50 enzymes à disposition était un défi majeur à relever. Ce développement est présenté dans un premier sous-chapitre dédié aux expériences préliminaires. En effet, un nombre considérable de stratégies de criblage a été envisagé, nous menant progressivement à des criblages effectifs. Ce sous-chapitre se focalise donc avant tout sur la démarche adoptée.

Le deuxième sous chapitre est rédigé sous forme d'une publication scientifique rédigée en anglais. Il décrit le criblage des 50 enzymes et la caractérisation de l'activité de deux de ces enzymes sur des polyuréthanes thermoplastiques.

Des expériences complémentaires décrivant la caractérisation des enzymes modifiées, aussi appelées mutants, sur des polyuréthanes thermoplastiques font l'objet d'un troisième sous chapitre. En effet, la séquence de l'une des deux enzymes sélectionnées à l'issue du criblage a été modifiée par Protéus afin d'améliorer son ancrage sur les substrats polymères et son activité hydrolytique envers la liaison uréthane.

Sous-chapitre 2.1. Développement de criblages enzymatiques

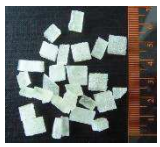
La première étape de ces travaux consistait donc à cribler la collection de 50 hydrolases fournies. Dans cette collection, 47 enzymes sont des estérases et 3 d'entre elles sont des amidases (E4142, E4143 et E4257). Chaque enzyme se présente sous la forme d'une solution non purifiée (6 mL) contenant des débris cellulaires. Le contrôle négatif utilisé dans les expériences ci-dessous correspond donc à une solution contenant les mêmes débris cellulaires que les solutions enzymatiques.

Plusieurs contraintes ont dû être prises en compte pour le criblage. Le nombre d'enzymes à cribler simultanément ainsi que le volume d'enzymes à disposition nous ont orientés vers le choix du format de la microplaque à 96-puits.

1. Criblage sur polyuréthanes thermoplastiques (TPU) broyés

Initialement, l'objectif était de cribler les enzymes sur des polyuréthanes thermoplastiques (TPU). Ces polymères synthétisés pour l'étude sont proches en termes de structures chimiques, de déchets de polyuréthanes à recycler et sont donc des modèles très réalistes. 4 TPU différents ont été synthétisés à partir de 4,4'-methylène diisocyanate (4,4'-MDI) et de 1,4-butanediol (1,4-BDO), la seule variable étant le polyol long (Tableau 2.1.1). Une attention particulière a été portée à la diversité de structures chimiques de ces polymères. Un TPU aliphatique modèle a aussi été synthétisé. Ce polymère aliphatique à base de groupes uréthanes ne contient aucun groupe chimique éther ou ester. Il permet ainsi d'évaluer l'activité enzymatique uniquement de la liaison uréthane.

Tableau 2.1.1 – Liste des polyols longs utilisés pour la synthèse des TPU

Polyols	Photos des TPU
Un polyester synthétisé à partir d'un dimère d'acide gras modifié	
Un poly(ester ether) synthétisé à partir d'acide téréphtalique et d'éthylène glycol	
Le polytetrahydrofurane, un polyether linéaire avec des terminaisons hydroxyles	
Le 1,12-dodecanediol (diol aliphatique)	

Pour mesurer les activités enzymatiques sur ces TPU, une première étape de broyage a été réalisée de manière à obtenir des suspensions opaques et homogènes. En effet, l'objectif était de disposer ces suspensions en microplaque et de suivre la dégradation des polymères par mesure de turbidité, en l'occurrence la clarification de la solution, à l'image de ce qui a été fait pour l'Impranil®.

Le broyage a été réalisé avec un mixeur en acier (Référence 7011HS, Waring commercial, Etats-Unis) avec ajout d'azote liquide. Les particules obtenues pour le poly(ester uréthane), le poly(ester éther uréthane) et le poly(éther uréthane) sont de l'ordre du millimètre. La mise en suspension dans un tampon aqueux (phosphate, 0,1 M, pH7) forme des agrégats pour le poly(éther uréthane) (Figure 2.1.1a) et le poly(ester uréthane). Une sédimentation rapide est

observée pour le poly(ester éther uréthane) (Figure 2.1.1b). Les particules du TPU aliphatique sont de l'ordre de 100 µm aussi bien avant qu'après broyage au mixeur. Ce polymère mis en suspension sédimente rapidement. L'hydrophobicité des TPU et la taille des particules obtenues empêchent la formation de suspensions homogènes.

Un second type de broyage a été mis en place. Un broyeur planétaire (Pulverisette 7, Fritsch) a été utilisé avec des billes de 3 mm pendant une minute à 700 rpm. La forte augmentation de température engendrée par ce broyage dénature le poly(ester uréthane), le poly(ester éther uréthane) et le poly(éther uréthane). En effet, une coloration brunâtre du polymère apparait. Pour le TPU aliphatique, une suspension d'aspect laiteuse a été obtenue (Figure 2.1.1c). Les particules de cette suspension sont alors de l'ordre de 30µm.

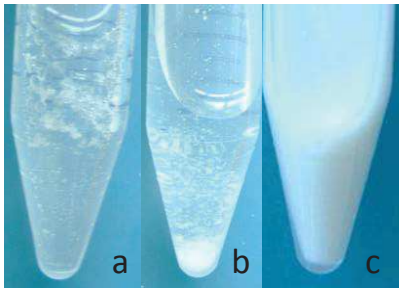


Figure 2.1.1 - Suspension de polyuréthanes broyés dans un tampon aqueux (a) du TPU base PTHF broyé au mixeur, (b) du TPU base acide téréphtalique et éthylène glycol broyé au mixeur et (c) du TPU base 1,12-dodecanediol broyé au broyeur planétaire

Ainsi, la suspension de TPU aliphatique a pu être disposée à une concentration de 5 g/L dans les puits d'une microplaque. Le criblage de l'intégralité de la collection d'enzyme a été réalisé. Aucune baisse d'absorbance n'a été observée après une incubation de 5 jours à 37°C. Plusieurs hypothèses peuvent expliquer ce résultat négatif. Il est possible qu'aucune des enzymes ne soit capable d'hydrolyser ce polymère de par la nature des liaisons chimiques, ou de par la cristallinité importante du polymère. Il est également possible que certaines activités potentielles soient non-détectables du fait de la faible sensibilité de la méthode. La réalisation d'un criblage sur TPU broyés par suivi de turbidité n'est donc pas concevable.

2. Criblage sur polyuréthane en dispersion aqueuse

Les polyuréthanes en dispersions présentent des atouts considérables pour l'évaluation d'activités enzymatiques par rapport aux TPU broyés. En effet, les dispersions sont synthétisées en phase aqueuse avec un émulsifiant qui apporte une couche hydrophile à la surface des particules. Cela peut notamment faciliter les interactions enzymes/polymères. La taille des particules est très réduite, généralement de l'ordre de la centaine de nanomètres. Dans cette partie, deux PU en dispersion ont été étudiés, un poly(ester uréthane) commercial, l'Impranil-DLN®, et un poly(ether uréthane).

2.1. Poly(ester urethane) en dispersion

Un premier essai a été réalisé dans un tube à essai contenant un tampon phosphate (pH7, 0,1 M) et 5 g/L d'Impranil®. La lipase de *Candida sp.* exprimée dans *Aspergillus niger* (Sigma L3170) a été utilisée comme contrôle positif pour ce test. En effet, des lipases issues du genre *Candida* ont déjà été décrites comme actives sur des poly(ester uréthanes) thermoplastiques (Li et al. 2015) et également sur l'Impranil® (Gautam, Bassi, and Yanful 2007). Aucun changement d'aspect n'est observé pour l'Impranil® incubé avec le contrôle négatif (Figure 2.1.2a et b). Après seulement quelques minutes d'incubation avec la lipase, la solution d'Impranil® se clarifie (Figure 2.1.2c et d). Afin d'augmenter le débit du criblage et de limiter le volume d'enzyme nécessaire, un test a été réalisé en déposant 2µL de lipase sur une boite de Pétri contenant un tampon phosphate pH7, 15 g/L d'agar et 1,5 g/L d'Impranil®. De la même manière qu'en tube, un halo de clarification apparait après seulement quelques minutes (Figure 2.1.2e). Comme cela est décrit dans la littérature, la clarification de l'Impranil® est très probablement le résultat de l'hydrolyse de liaisons esters et non celle des fonctions uréthanes. Ce criblage n'est donc pas suffisant pour la sélection d'enzymes dépolymérisant les polyuréthanes.

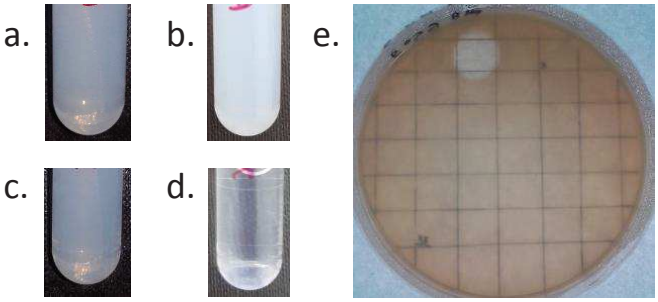
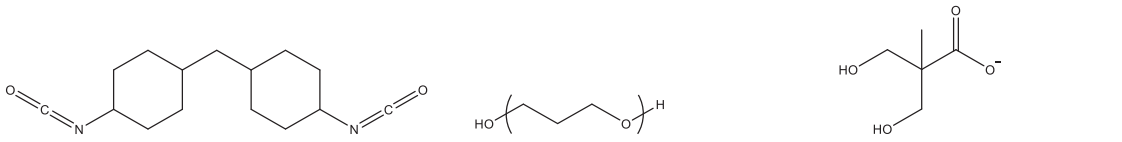


Figure 2.1.2 – Réaction entre le contrôle négatif et l'Impranil® (a) avant incubation (b) après incubation. Réaction entre la lipase et l'Impranil® (c) avant incubation (d) après incubation. (e) Hydrolyse de l'Impanil® par la lipase sur boite de Pétri

2.2. Poly(éther urethane) en dispersion

Un poly(éther uréthane) en dispersion aqueuse (PUD1 Sop) a été synthétisé. Ce polymère est obtenu à partir d'un isocyanate : le 4,4'-dicyclohexylméthane diisocyanate (H_{12}MDI), d'un polyol : le polytétrahydrofurane (PTHF) et d'un émulsifiant : l'acide 2,2-Bis(hydroxyméthyl)propionique (DMPA) (Figure 2.1.3). Les deux fonctions hydroxyles du DMPA réagissent avec le H_{12}MDI pour former des liaisons uréthanes. Le groupe carboxylique permet la mise en place d'une couche hydrophile autour des particules.



4,4'-Dicyclohexylmethane diisocyanate (H_{12}MDI)

Polytétrahydrofurane (PTHF)

Acide 2,2-Bis(hydroxyméthyl)propionique (DMPA)

Figure 2.1.3 – Composés utilisés lors de la synthèse de PUD1 Sop

Toutes les enzymes ont été criblées sur boite de Pétri dans les mêmes conditions que pour l'Impranil®. Aucun halo de clarification n'a été observé. Afin d'approfondir ces résultats, un test en microplaque a été réalisé. Les trois amidases ont été utilisées comme références pour ce test même si ne sont présentés que les résultats obtenus pour l'enzyme E4143. Les enzymes de cette famille ont déjà été décrites dans la littérature comme hydrolysant la liaison uréthane (Guo et al. 2014; Gamerith et al. 2016). Une baisse d'absorbance à 630 nm est observée pour PUD1 Sop

incubé avec le contrôle négatif et l'enzyme E4143 (Figure 2.1.4). En revanche, ni la lipase, ni le tampon n'entraine de baisse d'absorbance. La clarification n'est donc pas le résultat d'une hydrolyse enzymatique. Il est possible que cette clarification soit le fait d'une agrégation des particules de PUD1 Sop avec les débris cellulaires présents dans le contrôle négatif et l'enzyme E4143. Comme la lipase commerciale, qui est purifiée, et le tampon n'en contiennent pas, l'absorbance reste stable au cours du temps. Un criblage par suivi d'absorbance sur le polymère PUD1 Sop n'est donc pas envisageable.

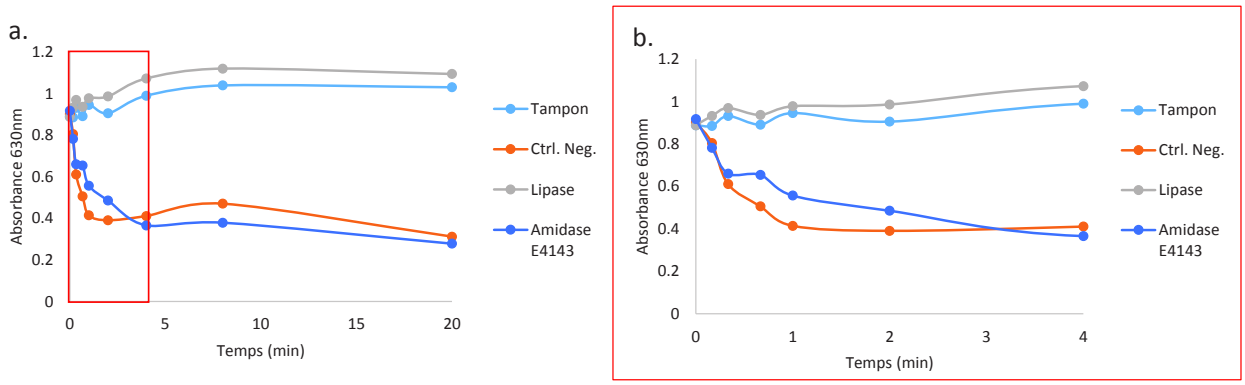


Figure 2.1.4 – Evolution de la turbidité (absorbance à 630 nm) du PUD Sop 1 incubé à 37°C (a) entre 0 et 20h et (b) entre 0 et 4h

3. Criblage sur molécules modèles de faibles masses molaires.

Le développement d'un criblage sur l'Impranil® n'est pas suffisant pour sélectionner une ou plusieurs enzymes dégradant les polyuréthanes. En effet, cela restreindrait les substrats PU aux seuls poly(ester uréthanes). Nous nous sommes donc focalisés sur le développement de criblages sur des molécules modèles de faibles masses molaires ne contenant pas de liaisons esters.

3.1. Mono-uréthane

Un mono-uréthane a été synthétisé à partir de p-toluenesulfonyl isocyanate (pTSI) et de butanol. L'hydrolyse de ce mono-uréthane entraine la production de p-toluenesulfonamide (pTSA), de dioxyde de carbone et la récupération du butanol (Figure 2.1.5a). Le protocole utilisé pour la réaction enzymatique en microplaque et l'analyse HPLC est présenté dans la suite de ce manuscrit

(Sous-chapitre 2.2.3.3.1, page 99). Comme pour le poly(éther uréthane) en dispersion, les résultats sont présentés pour l'amidase E4143. Des prélèvements ont été réalisés après 1, 2, 4 et 24h de réaction. Une baisse de la concentration en mono-uréthane est observée avec l'enzyme E4143 alors que le mono-uréthane incubé avec le contrôle négatif présente une concentration constante (Figure 2.1.5b). De la même façon, une augmentation du pTSA est observée pour la réaction avec E4143 alors qu'une très faible concentration apparait pour le mono-uréthane incubé avec le contrôle négatif (Figure 2.1.5c). Ce test permet donc d'évaluer la capacité d'une enzyme à hydrolyser la liaison uréthane. La réaction est réalisée en microplaque permettant un débit adapté à notre collection d'enzymes. De plus, cette expérience préliminaire confirme que l'amidase E4143 de la collection est capable d'hydrolyser la liaison uréthane.

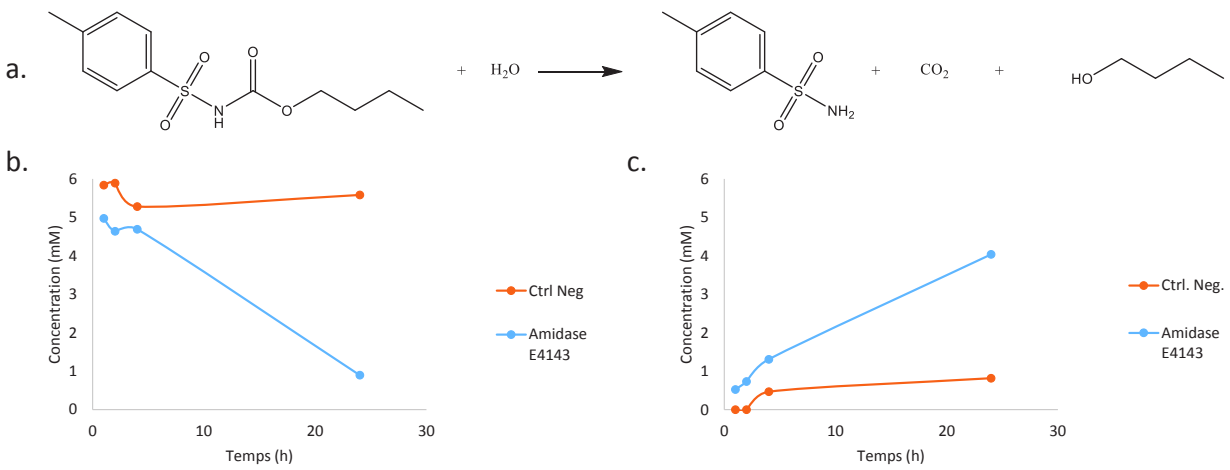


Figure 2.1.5 – (a) Hydrolyse enzymatique du mono-uréthane. (b) Evolution de la concentration en mono-uréthane au cours du temps (c) Evolution de la concentration en produit de dégradation (pTSA) au cours du temps

3.2. Oligo-uréthane

Pour tenter de détecter des activités sur la liaison uréthane d'une molécule plus complexe que le mono-uréthane mais moins complexe que les TPU ou le poly(éther uréthane) en dispersion. Pour ce faire, un pré-polymère de masse molaire limitée a été synthétisé avec du 4,4'-MDI et deux diols différents : le poly(propylène glycol) et le tripropylène glycol (Figure 2.1.6). L'agent d'extension de chaînes utilisé n'est pas un diol comme c'est le cas classiquement pour les TPU. Les isocyanates du pré-polymère ont réagi avec du butanol, qui ne contient qu'un groupement

hydroxyle. Cela a pour effet de bloquer l'allongement des chaines et de synthétiser un poly(éther uréthane) très court (entre 2 et 6 liaisons uréthanes). L'oligo-uréthane est donc composé de polymères de différentes tailles dont la masse molaire varie en fonction du nombre de liaisons uréthanes.

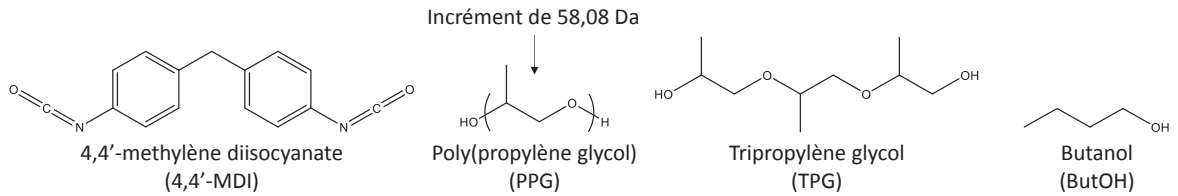


Figure 2.1.6 – Composés utilisés pour la synthèse de l'oligo-uréthane

Cet oligo-uréthane a un aspect visqueux ce qui permettrait de faciliter les interactions enzymes/polymères. De plus, il est possible d'analyser aisément cet oligomère en MALDI-TOF/MS (Figure 2.1.7). Les analyses ont été réalisées en utilisant du dithranol à 10 g/L solubilisée dans du tetrahydrofurane (THF) comme matrice. Le spectre MALDI-TOF/MS du poly(propylène glycol) présente une distribution unique avec des incréments de 58 Da correspondant bien à une unité propylène glycol (Figure 2.1.7a). En comparaison, plusieurs massifs de masses molaires plus importantes avec également un incrément de 58 Da apparaissent sur le spectre MALDI-TOF/MS de l'oligo-uréthane confirmant la réussite de cette synthèse.

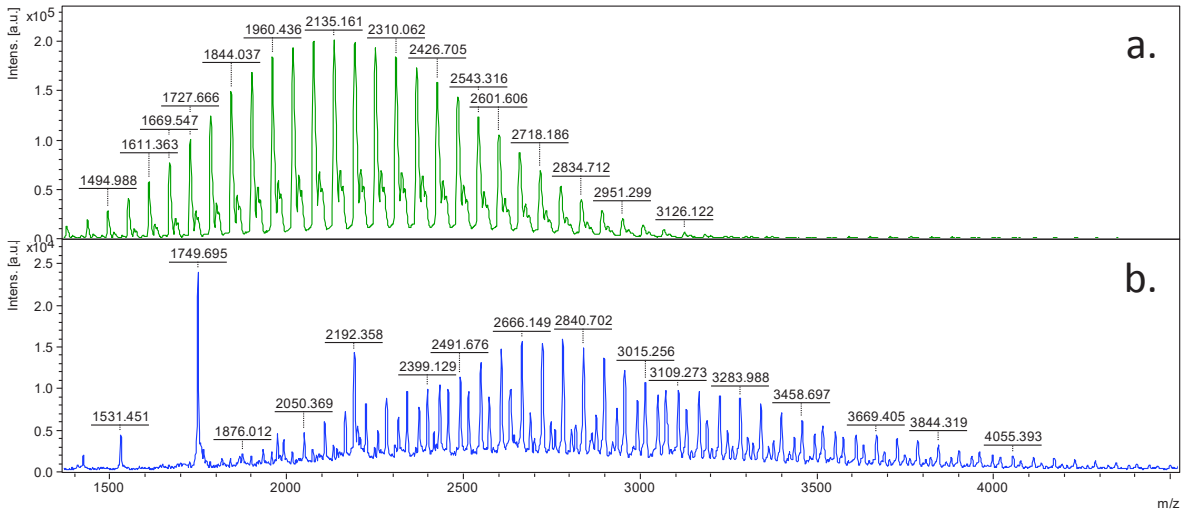


Figure 2.1.7 – Spectre MALDI-TOF/MS (a) du poly(propylène glycol) et (b) de l'oligo-uréthane

Une hydrolyse chimique a été testée pour vérifier qu'il est possible de détecter l'hydrolyse de l'oligo-uréthane par MALDI-TOF/MS. Les réactions ont été réalisées dans des tubes à essais en verre de 7 mL. Pour cela, 20 mg d'oligo-uréthane ont été déposés dans le fond des tubes, puis recouverts de 100 µL d'hydroxyde de sodium (NaOH) à 0,1 M et 0,5 M. Les tubes ont ensuite été incubés à 50°C pendant 18h. Après réaction, la fraction soluble a été retirée. La fraction solide correspondant à l'oligo-uréthane a été solubilisée dans du THF puis analysée par MALDI-TOF/MS. Une légère tendance se dégage montrant une augmentation des chaînes polymères de faibles masses molaires pour l'oligo-uréthane incubé avec le NaOH 0,5 M (Figure 2.1.8).

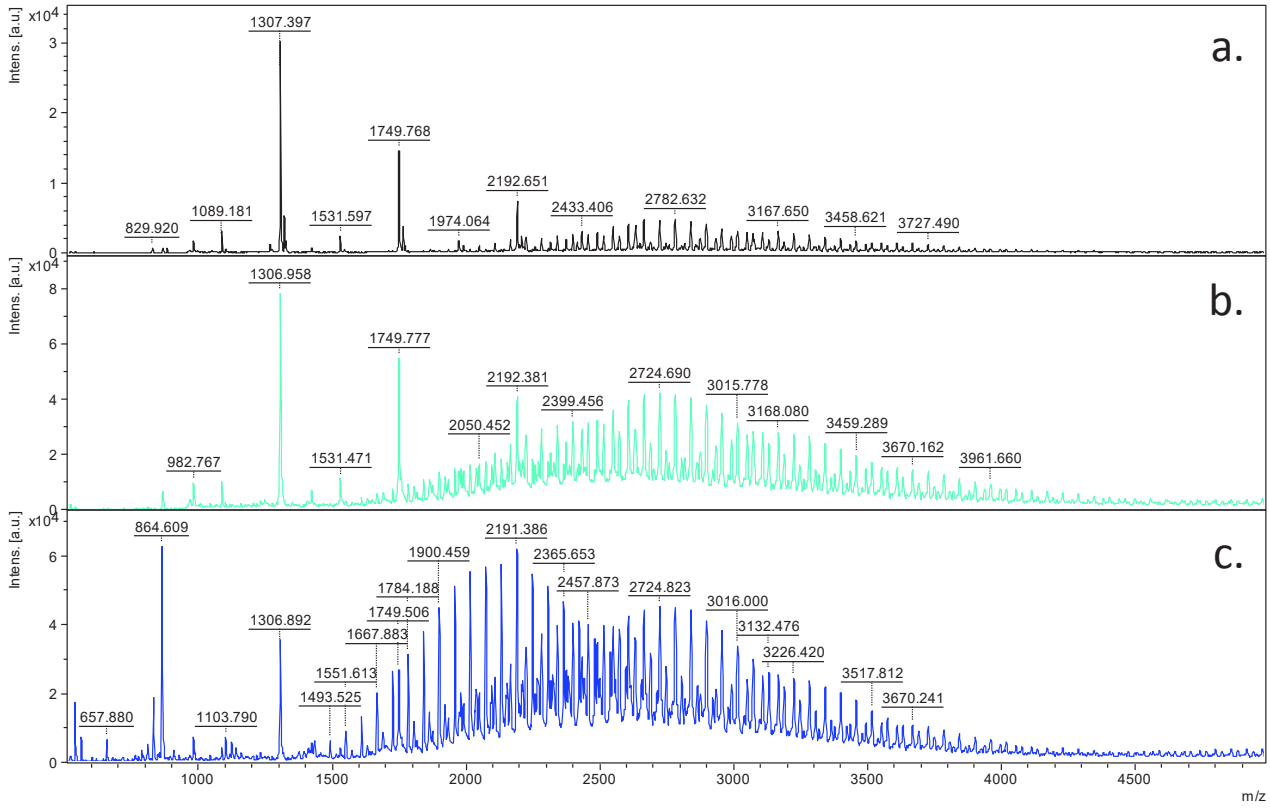


Figure 2.1.8 – Spectres MALDI-TOF/MS de l'oligo-uréthane après hydrolyse (a) avec le contrôle négatif (b) avec NaOH 0,1M (c) avec du NaOH 0,5M

Bien qu'une tendance soit observée, il est difficile d'expliquer clairement les évolutions des distributions de chaînes polymères sur les spectres. Un traitement des données spécifique a permis de grouper les différents massifs en prenant en compte les incréments de 58 Da qui correspondent aux unités propylène glycol (Figure 2.1.6). Ainsi, chaque courbe apparaissant sur

les graphiques (Figure 2.1.9) correspond à un oligo-uréthane contenant un nombre fixe de liaisons uréthanes. Après hydrolyse avec le NaOH 0,1 M, on observe l'apparition d'un polymère de plus faible masse molaire, contenant donc moins de liaisons uréthanes (Figure 2.1.9b, courbe orange). L'incubation de l'oligo-uréthane avec le NaOH 0,5 M montre l'apparition du même polymère de faible masse molaire que pour le NaOH 0,1 M, en abondance relative plus importante par rapport aux autres distributions. Ce traitement de données permet de confirmer l'hydrolyse de l'oligo-uréthane avec le NaOH 0,5 M ainsi que de montre un début d'hydrolyse pour le polymère incubé avec le NaOH 0,1 M.

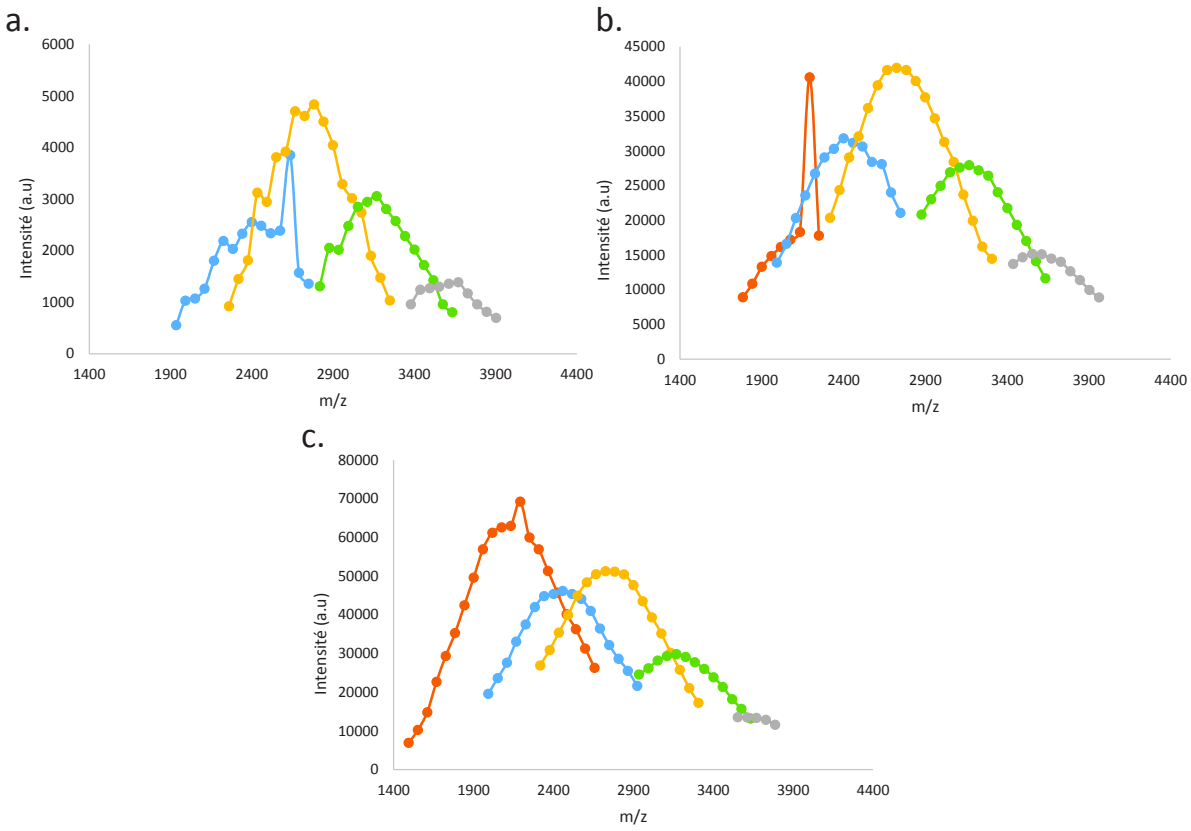


Figure 2.1.9 – Distribution en masse des polymères composant l'oligo-uréthane après hydrolyse (a) avec le contrôle négatif (b) avec NaOH 0,1M (c) avec du NaOH 0,5M

La réaction a ensuite été transposée à l'hydrolyse enzymatique. Les réactions se sont déroulées en tubes comme précédemment. 100 µL d'enzyme E4143 ont été ajoutés directement sur le polymère. Des profils similaires sont obtenus en MALDI-TOF/MS pour l'oligo-uréthane incubé

avec le contrôle négatif et l'enzyme E4143 (Figure 2.1.10). Différentes conditions de réactions ont été testées (ajout de tampon, températures entre 30 et 50°C, temps d'incubation entre 4 et 24h, volume de solution enzymatique entre 100 et 300 µL). Aucune différence n'a pu être établie entre le contrôle négatif et l'enzyme E4143, pour aucune des conditions testées. De plus, d'autres enzymes de la collection ont été testées sans montrer d'activité. Puisque l'analyse MALDI-TOF/MS de l'oligo-uréthane permet de détecter une hydrolyse, comme l'a montré l'hydrolyse chimique par une base, il semble donc que l'enzyme E4143 et les autres enzymes testées soient inactives sur ce substrat. D'après les expériences menées sur le mono-uréthane, l'enzyme E4143 est capable d'hydrolyser la liaison uréthane. Il semble donc que la structure polymère de l'oligo-uréthane le rende insensible à l'hydrolyse enzymatique par E4143. L'utilisation de cette méthode pour le criblage des 50 enzymes n'est donc pas envisageable.

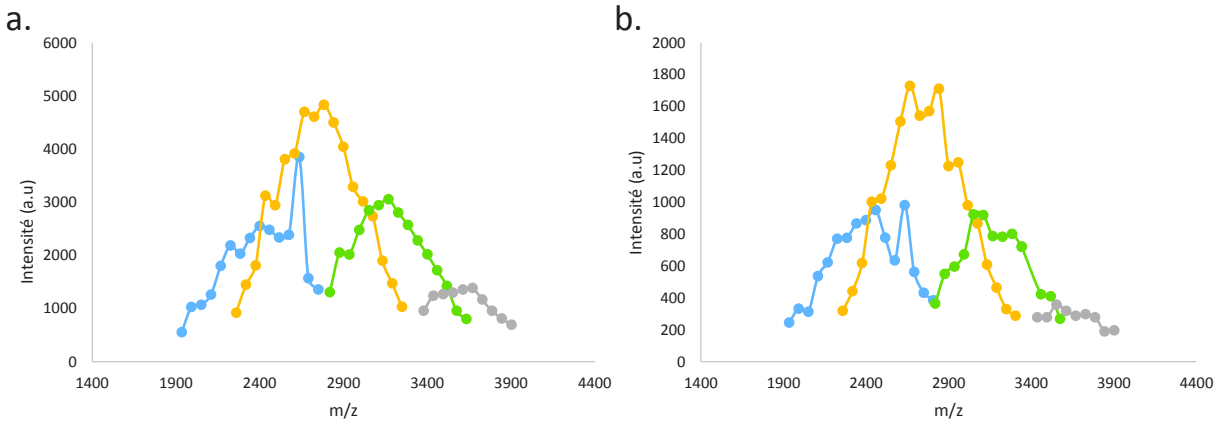


Figure 2.1.10 – Distribution en masse des polymères composant l'oligo-uréthane après hydrolyse (a) avec le contrôle négatif (b) avec l'enzyme E4143

4. Conclusion

Parmi les différents criblages envisagés pour la sélection d'enzymes dépolymérisant les PU, deux criblages permettent d'évaluer des activités enzymatiques. Le criblage sur le polyuréthane en dispersion commercial Impranil-DLN® permet de détecter des activités sur un poly(ester uréthane). Ces tests ont été réalisés sur boite de Petri par clarification puis confirmer par mesure d'absorbance dans une microplaque à 96 puits. Cependant, ce criblage permettra uniquement la

détection des enzymes actives sur des liaisons esters. En complément, un criblage par HPLC sur une molécule modèle, également en microplaque à 96-puits, ne contenant qu'une liaison uréthane permet de détecter des activités sur cette liaison spécifique.

Avec ces deux types de criblages effectifs, il nous est désormais possible de cribler la collection d'enzymes mise à disposition. Ceci fait l'objet du sous chapitre suivant.

5. Références

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Sous-chapitre 2.2. Enzymatic recycling of thermoplastic polyurethanes: synergistic effect of an esterase and an amidase and recovery of building blocks

Audrey Magnin[1], Eric Pollet[1], Rémi Perrin[2], Christophe Ullmann[3], Cécile Persillon[3], Vincent Phalip*[,4], Luc Avérous*[,1]

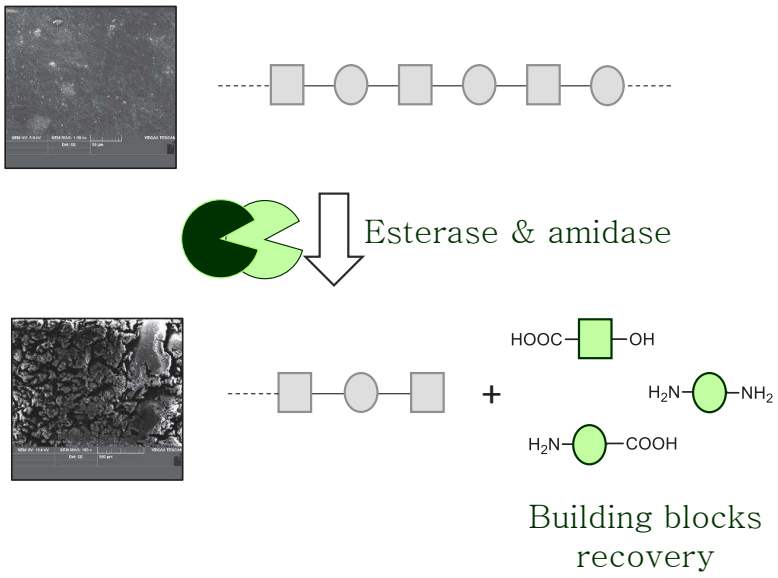
(1) BioTeam/ICPEES-ECPM, UMR CNRS 7515, Université de Strasbourg, 25 rue Becquerel, 67087 Strasbourg Cedex 2, France

(2) SOPREMA, 14 rue de Saint-Nazaire, 67025 Strasbourg, Cedex 1, France

(3) Proteus S.A., 70 allée Graham Bell, Parc Georges Besse, 30035 Nîmes Cedex 1, France

(4) Univ. Lille, INRA, ISA, Univ. Artois, Univ. Littoral Côte d'Opale, EA 7394 - ICV - Institut Charles Viollette, 59000 Lille, France

(*) Corresponding authors: luc.averous@unistra.fr



1. Abstract

Biological recycling of polyurethanes (PU) is a huge challenge to take up in order to reduce a large part of the environmental pollution from these materials. However, enzymatic depolymerization of PU still needs to be improved to propose valuable and green solutions. The present study aims to identify efficient PU degrading enzymes among a collection of 50 hydrolases. Screenings based on model molecules were performed leading to the selection of an efficient amidase (E4143) able to hydrolyze the urethane bond of a low molar mass molecule and an esterase (E3576) able to hydrolyze a waterborne polyester polyurethane dispersion. Degradation activities of the amidase, the esterase and a mix of these enzymes were then evaluated on four thermoplastic polyurethanes (TPU) specifically designed for this assay. The highest degradation was obtained on a polycaprolactone polyol-based polyurethane with weight loss of 33 % after 51 days measured for the esterase. Deep cracks on the polymer surface observed by scanning electron microscopy and the presence of oligomers on the remaining TPU detected by size exclusion chromatography evidenced the polymer degradation. Mixing both enzymes led to an increased amount of urethane bonds hydrolysis of the polymer. 6-hydroxycaproic acid and 4,4'-methylene dianiline were recovered after depolymerization as hydrolysis products. Such building blocks could get a second life with the synthesis of new macromolecular architectures.

Keywords: Enzymatic degradation, polyurethane, hydrolase, polymer pollution, bio-recycling

2. Introduction

Enzymatic depolymerization of synthetic polymers is an appealing approach to reduce a large part of the environmental pollution from these materials and to address waste management issues in a green way. This soft process is considerably more eco-efficient than chemical ones as it takes place at moderate temperatures without chemical catalysts and is then less polluting. Furthermore, controlled enzymatic depolymerization generates building blocks which can be valorized to develop new macromolecular architectures, for example. This approach can be considered as a promising biotechnical recycling process. A prominent example is the recycling of polyesters, particularly for poly(ethylene terephthalate) (PET), that recently led to an efficient depolymerization process of PET-based packaging (Gamerith et al. 2017).

Polyurethanes (PU) are equivalent to PET in terms of plastic consumption in Europe (Plastic facts 2016), with 7.5 versus 7.1% for PU and PET, respectively. Since Otto Bayer's process in 1937, PU are mainly obtained by the polyaddition between polyols and polyisocyanates (Debuissy, Pollet, and Avérous 2017). Urethane group can be considered as the combination of an ester and an amide bond. PU contain amorphous regions, known as soft segments, principally based on long polyester- or polyether-based polyols, and organized hard segments, consisting of the isocyanate groups and the chain extender moieties based on short molecules (Figure 2.2.1) (Laurichesse, Huillet, and Avérous 2014).

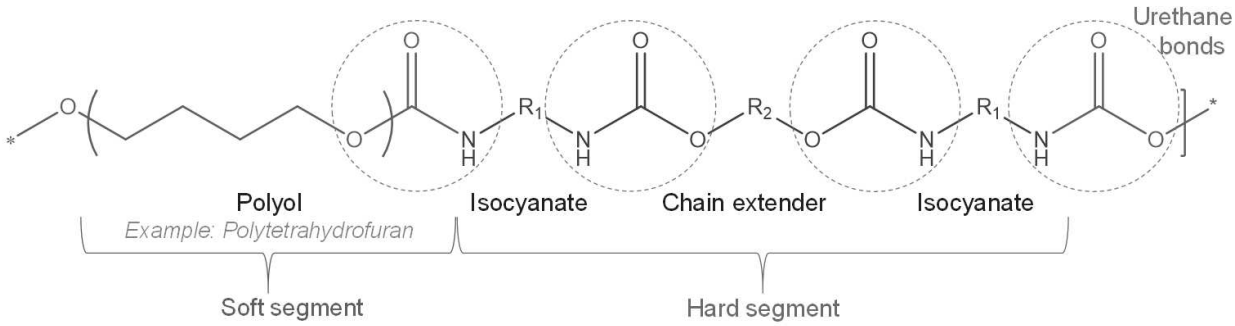


Figure 2.2.1 – Example of the chemical structure of a thermoplastic polyurethane

Materials properties and behavior can be largely modulated by varying the chemical nature and content of the soft and the hard segments. PU, ranking 6th among polymer worldwide production, are thus an attracting polymer family with a great diversity of macromolecular architectures (Furtwengler, Boumbimba, and Avérous 2018). Main markets are foams (65%), coating (13%), adhesives sealant (7%), and elastomer (12%) for a global production of 18 MTons in 2016 (Furtwengler et al. 2017). PU are mainly used for long-term applications, since they are highly resistant due to the strong urethane bonds and their specific micro-organizations and morphologies. The persistent presence of PU in the environment is a direct consequence of PU's resilient material properties in combination with inadequate waste management systems. A recent study showed that among 70 pieces of foamed plastic material collected on a British beach, 39 were identified as PU (Turner and Lau 2016).

Enzymatic depolymerization of PU has mostly been studied on poly(ester urethane). Esterases such as lipases (EC 3.1.1) (Schöne et al. 2016; Fang et al. 2014), cutinases (EC 3.1.1.74) (Yang et al. 2013; Schmidt et al. 2017) or unspecified esterases (EC 3.1) (Kang et al. 2011) have been used for this purpose. PU degrading activity is often evaluated on a commercial waterborne poly(ester urethane) dispersion (PUD), Impranil® (Covestro, Germany) (Crabbe et al. 1994). This colloidal polymer, considered as a model, has a milky aspect and turns translucent once degradation takes place. As highlighted by Biffinger *et al.* (Biffinger et al. 2015), this "clarification" results from the hydrolysis of the ester bonds of the soft segment without any evidence of urethane bond cleavages. This aliphatic polyester-polyurethane dispersion can be considered as a model but its chemical architecture is very specific and not representative of most common PU. Indeed, as the 4,4'methylene diisocyanate (4,4'-MDI) is the most widely used isocyanate and is responsible for material resistance, 4,4'-MDI-based PU are more representative of actual PU waste. Experiments on thermoplastic polyurethane (TPU) based on this isocyanate seem thus more relevant. For example, poly(propylene succinate) and poly(butylene succinate)-based TPU containing 4,4'-MDI displayed a weight loss of 25 and 5%, respectively, after 7 weeks of incubation with a *Candida* lipase (Li et al. 2015). These results emphasize the slowness of the biodegradation of 4,4'-MDI-based TPU.

A large part of PU production is based on polyether polyols but only few publications deal with the enzymatic biodegradation of poly(ether urethanes). For instance, hydrolysis of a poly(ether urethane) based on 1,6-hexamethylene diisocyanate (HMDI) and triethylene glycol was observed with two proteolytic enzymes, papain (EC 3.4.22.2) and chymotrypsin (EC 3.4.21.1). However, no activity was observed using two esterases, a lipase and a cholesterol esterase (EC 3.1.1.13) under the same conditions (Campinez et al. 2013). A poly(ether urea urethane) based on 4,4'-MDI was hydrolyzed by both papain and urease (EC 3.5.1.5) (Marchant et al. 1987). But, to the best of our knowledge, no enzymatic degradation of 4,4'-MDI based TPU only based on a polyether-polyol has been published up to now. Substrates wherein the urethane bond is the only cleavable linkage were prepared to evaluate the specific activity of enzymes on urethane bonds. Akutsu-Shinego *et al.* (Akutsu-Shigeno et al. 2006) synthesized low molar mass urethanes and evaluated their enzymatic hydrolysis by GC/MS analysis of the degradation products. The synthesis of 1-methoxypropan-2-yl(4-nitrophenyl) carbamate allowed the measurement of enzymatic activity by measuring the release of 4-nitroaniline by absorbance at 405 nm (Gamerith et al. 2016).

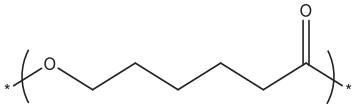
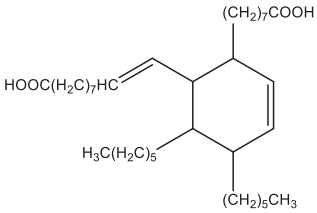
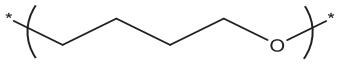
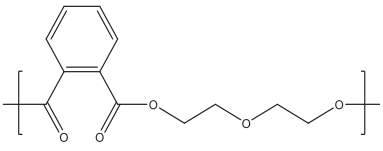
In this work, a collection of 50 hydrolases was screened with two model substrates: (i) Impranil® to evaluate the activity on polyester PU and (ii) a low molar mass substrate with a single urethane bond as the cleavable linkage. Depolymerization capacities of the best candidates were then evaluated on 4,4'-MDI-based TPU. In order to cover a wide range of PU, both poly(ester urethanes) and poly(ether urethanes) were studied. Weight loss, molar mass decrease and microscopic analysis were combined to obtain a complete overview of the degradation of TPU pieces. Recovery and analysis of the main degradation products by NMR was then performed. To evaluate the urethane bond hydrolysis, 4,4'-methylene dianiline (MDA) resulting from the hydrolysis of 4,4'-MDI-based PU was quantified using advanced techniques based on mass spectrometry.

3. Material and methods

3.1. Chemicals, reagents and enzymes

The various polyols used for TPU synthesis are described in Table 2.2.1. The poly(ester urethane) dispersion was Impranil® DLN-SD, kindly provided by Covestro (Germany) The p-toluenesulfonyl isocyanate (Luna PTSI – M = 197.2 g/mol) was purchased from DKSH (Switzerland). The 4,4'-methylene diphenyl diisocyanate (4,4'MDI, Suprasec 1306 - M = 250.25 g/mol) was purchased from Huntsman (USA). The 1,4-butanediol (1,4-BDO, M = 90.12 g/mol) was purchased from Invista (USA). Common reagents and solvents were purchased from Sigma (France) and used as received.

Table 2.2.1 – Polyols used for thermoplastic polyurethane synthesis

Commercial name	Supplier of the polyols	Composition	Chemical structure of the main components of the polyols
Capa 2302	Perstorp	Polyester: polycaprolactone (PCL)	
Radia 7285	Oleon	Polyester based on fatty acid dimer from rapeseed oil and short diol	
Terathane 2000	Invista	Polyether: Polytetrahydrofuran (PTHF)	
Stepanpol PD56	Stepan	Polyester based on diethylene glycol and phthalic anhydride	

A collection of 50 commercially available hydrolases was kindly provided by Protéus (France). These enzymes are esterases except E4142, E4143 and E4257 that are amidases. These enzymes were provided as enzymatic solutions corresponding to supernatants of crude extracts of *Escherichia coli* strains overproducing the required enzymes. As enzymes were not purified, a negative control solution has been prepared following the same protocol as the one for the enzymatic solutions but from a strain expressing no recombinant enzyme. A lipase from *Candida sp.* expressed in *Aspergillus niger* was purchased from Sigma (Sigma L3170).

3.2. Chemical synthesis of the substrates

The mono-urethane (MU) synthesis was performed in a 100 mL round-bottomed flask equipped with a magnetic stirrer and a nitrogen gas bubbling system. p-toluenesulfonyl isocyanate with a isocyanate group (NCO) was added followed by the butanol with one hydroxyl group (OH) to obtain stoichiometric conditions with NCO/OH ratio of 1.

The TPU synthesis was performed into a jacketed reactor, heated at 80°C, and equipped with a mechanic stirrer, and a nitrogen gas flow. Stoichiometric conditions were also used with a NCO/OH ratio of 1, using an equimolar quantity of the long polyol and 1,4-BDO. A “one-step” method was performed. The diisocyanate, the 4,4’-MDI, was warmed at 60°C and then introduced into the reactor followed by the mix of long polyol and 1,4-BDO, which was heated at 80°C beforehand. Resulting TPU material was then compressed at 145°C with a plate press (Labtech Hot Press) to obtain 0.3 mm thin films. Films were cut into pieces of 50-80 mg, 0.5x0.5 cm^2.

3.3. Degradation assays

3.3.1. MU screening

MU was solubilized into dimethyl sulfoxide (DMSO) to a final concentration of 500 µM. In a 96 well-microplate, 0.5 µL of this solution was added to 20 µL of enzymatic solutions and 29.5 µL of Tris-HCl buffer (50 mM, pH 7). Incubation was performed at 37°C and stopped after 40h by diluting reaction media at 1/20 in acetonitrile. Samples were filtered at 0.2 µm before HPLC analysis. Column used was a ACE 5 C8-300 250 x 3.0 mm (ACE) with H_2O containing 0.1% formic

acid and acetonitrile as mobile phase. A gradient from 15 to 55 % of acetonitrile (9 minutes) was applied with a flow of 1 mL/min. Absorbance was measured at 230 nm. MU and p-toluene sulfonamide (pTSA) were quantified with standards ranging from 2 to 5 mM (Figure S2.2.9). Reactions were first performed as singleton then in triplicate for the validation assay. Student t-test was performed with a p-value of 0.03 for the validation assay.

3.3.2. PUD screening

The PUD used for this assay was Impranil® DLN-SD. Phosphate buffers (0.1 M, pH 6 and 8) and carbonate buffer (0.1 M, pH 10) were supplemented with 2 g/L of PUD and 15 g/L of agar and poured on grid plates. Two microliters of enzymatic solutions were spotted on the plate. PUD clarification was evaluated qualitatively after overnight incubation at 30, 45 and 65°C. A positive control consisting in the lipase from *Candida sp.* expressed in *Aspergillus niger* was used. Activities of *Candida* lipase have already been described for the PUD (Li et al. 2015; Gautam, Bassi, and Yanful 2007). For the microplate assay, 20 µL of enzymatic solutions were added to 80 µL of phosphate buffer (0.1 M, pH 7). Absorbance was measured at 600 nm (A600) for five minutes. Activity was expressed as the decrease of $A600.min^{-1}.mL\ of\ enzymes^{-1}$. This assay was performed at 37 °C and pH 7 to remain on the same conditions than the MU screening.

3.3.3. TPU degradation assay

The TPU pieces were washed twice with distilled water, once with 70% ethanol and dried overnight at 37°C before being introduced in 4 mL glass tubes. To 900 µL of phosphate buffer (0.1 M, pH 7) were added 100 µL of enzymatic solutions. The solution was replaced every 3 or 4 days to overcome a loss of enzymatic activity. The liquid fractions were collected and stored at -20°C for degradation products analysis by liquid chromatography/electrospray/time-of-flight mass spectrometry (LC/ESI/qTOF-MS). Pieces were weighed before each addition of fresh enzymatic solutions. For that, TPU pieces were washed twice with distilled water, once with 70% ethanol and allowed to dry overnight at 37°C before weighing. Degradation reactions were performed over 51 days.

For NMR analysis of degradation products, reactions were performed in the same conditions but stopped after 3 days of incubation. The liquid fractions of the three repetitions were collected

and pooled together. The distilled water and the ethanol 70% solutions used to wash the TPU pieces were added to this liquid fraction to ensure the full recovery of the degradation products. Washed TPU pieces were weighed. The whole volume recovered was then dried under vacuum at 40°C. Dried products were solubilized in chloroform, filtered at 0.2 µm and dried again under vacuum at 40°C. This purification step was added to remove remaining enzymes and salts that are not soluble in chloroform. The recovered products were finally weighed.

3.4. Characterization techniques

Scanning electronic microscopy (SEM) was used to study the evolution of the surface morphologies of the degraded films. Vega-3 (Tescan) apparatus in high vacuum mode with an acceleration voltage of 5 kV and working distances in the range of 6-8 mm was used. Before examination, samples were coated with a thin layer of gold using a sputter coater (Quorum Q 150 RS, Quorum Technologies).

Size exclusion chromatography (SEC) was used to determine the evolution of the polymers molar masses. TPU was dissolved in tetrahydrofuran (THF) at room temperature and filtered with 0.2 µm PTFE filters directly in vials. SEC analyses were performed with an Acquity-APC (Waters) in THF at 40 °C at a flow rate of 0.6 mL/min. Three columns (Acquity APC XT 450 Å 2.5 µm 4.6x150 mm, 200 and 45) were connected. 10 µL of dissolved polymer solution were injected. A calibration curve using linear polystyrene (PS) standards was built and used for molar mass determination.

^{1}H NMR was used to study the chemical structures of the main degradation products. All the purified degradation products recovered were solubilized in 600 µL of deuterated chloroform ($CDCl_3$). Spectra were recorded using Bruker UltraShield 400MHz at room temperature. The relaxation delay was set to 10 s with 32 scans. Spectra were calibrated using the $CDCl_3$ peak at 7.26 ppm.

The analysis of 4,4'-MDI based degradation products has been performed by LC/ESI/qTOF-MS. Liquid fractions collected every 3 or 4 days were defrosted and centrifuged 5 min at 12 000 g. The supernatants were pooled together. The solution was concentrated under vacuum at 40 °C for 6h then, an equivalent volume of methanol was added for protein precipitation. Samples were filtered at 0.2 µm directly in vials before analysis. Minor modifications were made to the analytical

procedure for the detection of 4,4' methylenedianiline (MDA) previously described in Gamerith *et al (Gamerith et al. 2016)*. Samples were separated by reversed-phase chromatography using an ACQUITY-biocompatible HPLC-system (Waters, United Kingdom) equipped with a Phenomenex C18 Kenetex EVO core-shell column (ID 3.0 mm×150 mm, 4.6 µm, 100 Å). A gradient elution system consisted of mobile phase (A) of 65 mM ammonium formate and mobile phase (B) of acetonitrile (containing 0.1% formic acid) with a flow rate of 0.75 ml/min. The gradient was initiated at 5% B, increased to 30% B at 8 min, to 40% B at 11 min and to 90% B at 19 min. The HPLC eluent was directly electrosprayed from the column end at an applied voltage of 3 kV, using a desolvation gas (N_2) flow of 600 L/h, a nebulizer gas flow of 6.5 bar and source and desolvation temperatures of 80 and 150 °C, respectively. The chromatographic system was coupled to a SYNAPT-G2-Si mass spectrometer (Waters) operating in data-dependent mode. The full MS survey scans were acquired at a 2,000 (FWHM) resolving power, over the mass range of 20–2,000 m/z. Precursors observed with an intensity over 5,000 counts were selected for ion trap CID fragmentation with an isolation window of 3 amu and a normalized collision energy of 20 V. The MS/MS spectra were recorded on the 20 to 2000 m/z range. A maximum injection time of 100 ms was used for CID MS2 spectra that were acquired over the same mass range. The method was set to analyze the most intense ions from the survey scan. Peaks were analyzed using Mass Lynx software (version 4.1, Waters Corporation-USA).

4. Results and discussion

4.1. Medium throughput screenings based on the MU and the PUD degradation

According to the literature, urethane bond hydrolysis leads to a primary amine, an alcohol and carbon dioxide (Marchant et al. 1987; Doddamani and Ninnekar 2001). The hydrolysis of MU thus should induce the release of p-toluenesulfonamide (pTSA) (Figure 2.2.2a). Both MU and pTSA can be quantified in a single HPLC run. Enzymatic reactions leading to a MU concentration below 3.5 mM (Figure 2.2.2b) or a PTSA release higher than 1.25 mM (Figure 2.2.2c) were selected for a round of validation, which comprises thirteen candidates (in black on the Figure 2.2.2b and c).

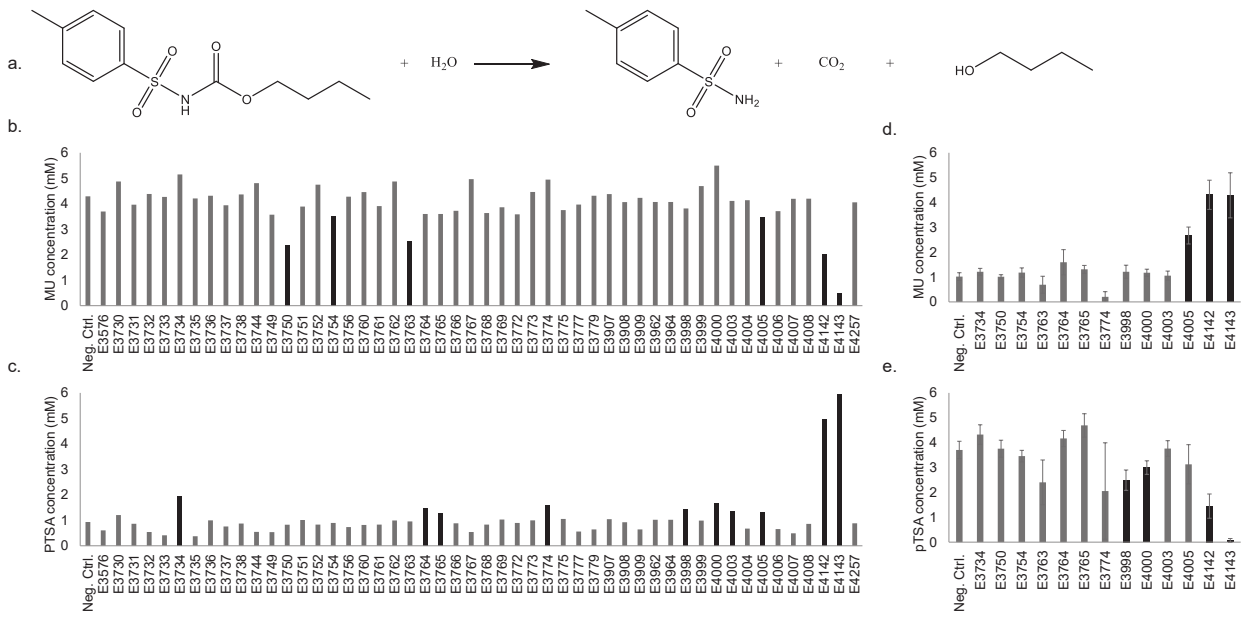


Figure 2.2.2 – (a) Hydrolysis of the MU leading to the release of pTSA. Concentration of (b) MU and (c) pTSA after 40h incubation at 37°C with the enzyme collection - Concentration of (d) pTSA and (e) MU of the candidate enzymes from the screening (n=3, test t-student with p<0.03)

Only two enzymes (E4142 and E4143) displayed both a significant (p<0.03) increase of pTSA concentration and a significant decrease of MU content (Figure 2.2.2d and e). E4143 seemed to be the most active as almost no residual MU was detected after reaction. E4143 is an amidase from Protéus. This result is in good agreement with previous publications on the effect of amidases on urethane bonds (Akutsu-Shigeno et al. 2006; Pérez-Lara et al. 2016). In addition, amidases have been described to hydrolyze polyamide chains (Acero et al. 2012; Guo et al. 2014). Very recently, degradation activity of an amidase was demonstrated on a model urethane substrate and on polyester PU pellets (Gamerith et al. 2016).

The screening on PUD was performed with agar plates containing PUD. Nine combinations of pH and temperatures were tested to maximize the positive hits. Four enzymes displayed PUD clarification after overnight incubation: E3777 (pH 8, 45 °C), E3769 (pH 6, 30 °C), E3576 (pH 8, 45 °C) and E3774 (pH 10, 65 °C) (Figure 2.2.3a). A quantitative method for measuring activity relying on absorbance decrease at 600 nm on microplate was then used to compare these four enzymes (Figure 2.2.3b). It revealed that E3576 has the highest clarification rate with a 5-time

higher rate than E3777, which is the second most active enzyme. E3576 is an esterase and this type of enzymes has been largely described for its polyester hydrolysis ability (Nomura et al. 1998; Stern and Howard 2000).

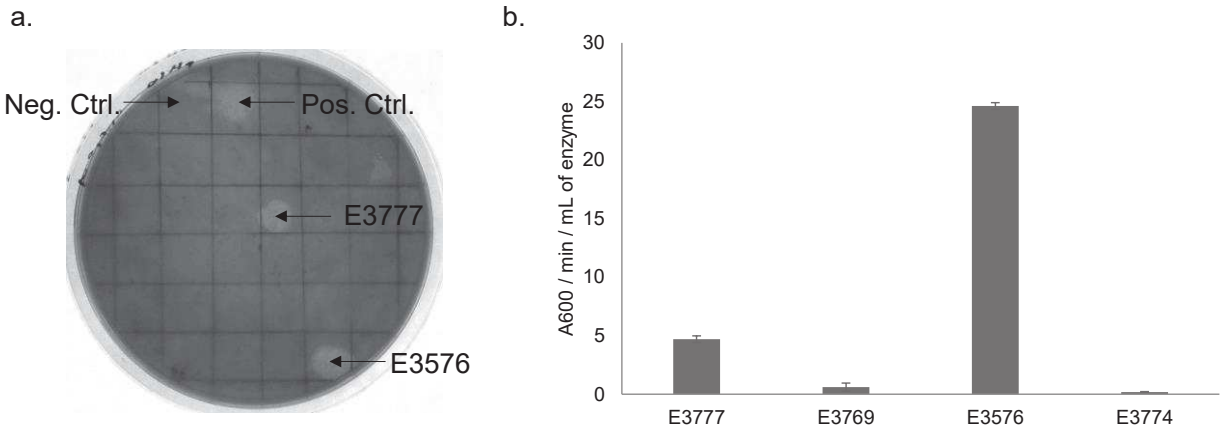


Figure 2.2.3 – (a) Screening on agar plate containing PUD (pH8 and 45°C) (b) activity measurement on microplate for the four candidate enzymes from the screening expressed as the decrease of absorbance at 600nm per minute per mL of enzymes

These two complementary screenings thus revealed 2 enzymes able to hydrolyze the urethane bond of a low molar mass model molecule and 4 enzymes able to hydrolyze PUD, a model polyester-based PU dispersion. The most active enzymes were selected: E3576 and E4143. They present two types of activity on urethane-based chemicals. The esterase E3576 was inefficient on the MU but hydrolyzed ester bonds of the PUD. Conversely, the amidase E4143 is not able to clarify the PUD. However, one cannot exclude that this amidase could hydrolyze urethane bonds of the PUD without noticeable clarification since E4143 is apparently not able to hydrolyze ester bonds of the PUD. Indeed, ester bonds content is largely higher than urethane bonds in PUD macromolecular architecture.

4.2. Study of the enzymatic degradation of TPU

After the screening, the selected enzymes were then evaluated on 4,4'-MDI-based TPU. These polymers were specially designed for the degradation assays. Their use presents a great advantage to understand the mechanisms of enzymatic degradation. For these assays, the tested TPU showed diverse architectures based on different long polyols to cover and represent a great variety of common PU structures and properties (Table 2.2.1). The four TPU present comparable average molar masses. They show number-average molar masses (Mn) higher than 34 kg/mol. Mass-average molar masses are higher than 75 kg/mol and dispersity (Đ) is between 2 and 3, attesting for successful synthesis of long TPU chains (Table S2.2.2). The negative control, the amidase E4143 and the esterase E3576 were tested alone and mixed together on these MDI-based TPU with the aim to target both urethane and ester bonds.

Enzymatic degradation kinetics of PU are slow (Schmidt et al. 2017) and thus require long reaction time. Then, to overcome the enzyme denaturation observed after a few days of incubation, the enzymatic solutions are frequently renewed in lengthy degradation experiments (Li et al. 2015). When commercial enzymes are employed, incubation of up to seven days may be performed without any activity loss (Tang, Labow, and Santerre 2003a; Guan, Fujimoto, and Wagner 2008). But, as the enzymes used in this study do not contain conservative agents, enzyme solutions were changed every 2 or 3 days.

Degradation kinetics can be evaluated by weighing TPU pieces before each addition of fresh enzymatic solution. A significant release of water soluble degradation products would lead to noticeable mass loss. No weight loss was observed for the fatty acid dimer-based TPU (Figure 2.2.4b), the poly(ether urethane) (Figure 2.2.4c) and the poly(ester-ether urethane) (Figure 2.2.4d). These polymers may be resistant to the enzymatic hydrolysis. It is also possible that the weight loss measurement was not sensitive enough to detect low degradation extents. In contrast, weight losses of 33 and 27% were measured for the PCL-based TPU incubated with the esterase E3576 alone and E4143/E3576 mix, respectively (Figure 2.2.4a).

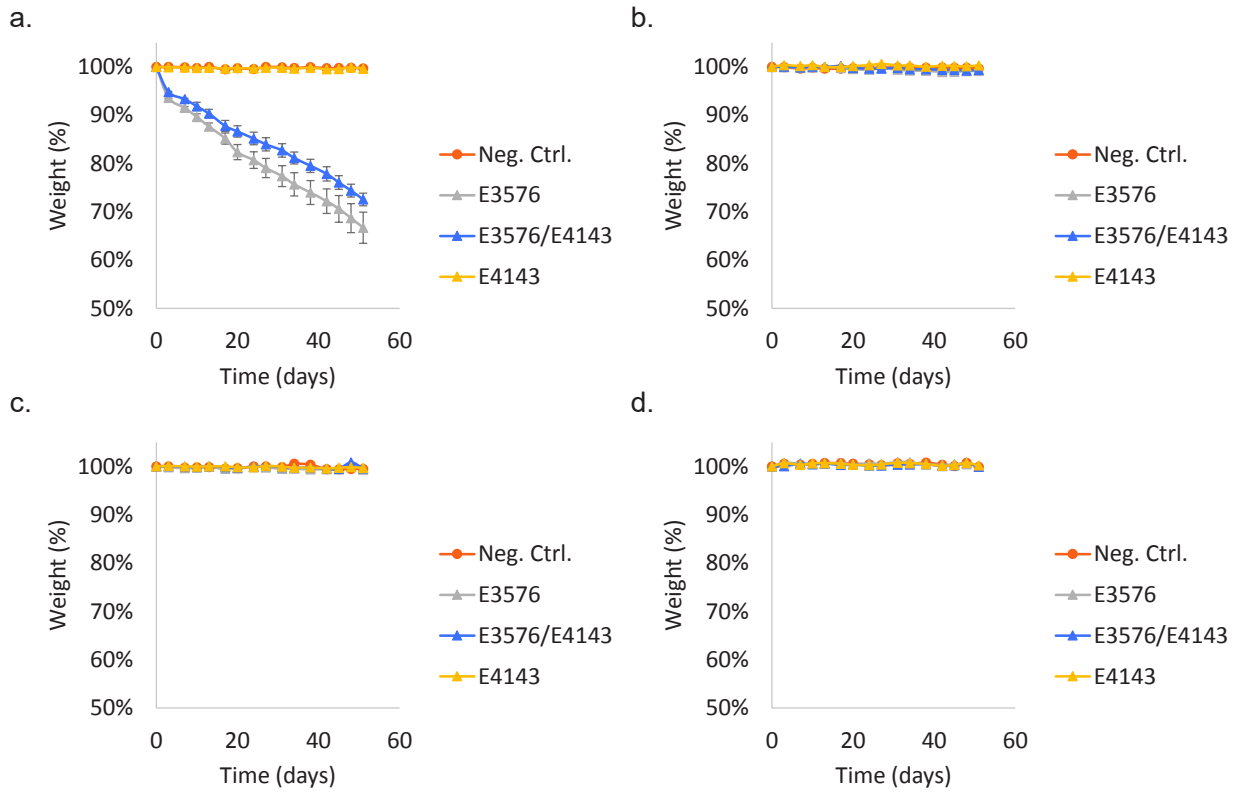


Figure 2.2.4 – TPU weight decrease on 51 days incubated at 37°C with enzymatic solutions (a) PCL-based TPU, (b) Fatty acid dimer-based TPU, (c) poly(ether urethane) and (d) poly(ester-ether urethane)

The degradation with the E4143/E3576 mix involved half of the volume of esterase E3576 compared to the degradation with the esterase alone. However, the degradation with the E4143/E3576 mix led to a weight loss close to the weight loss measured with the esterase E3576 alone and not half of it. To assess if the amidase E4143 could have played a specific role in the degradation with the E4143/E3576 system, a three-day incubation with different volumes of esterase E3576 completed to 100 µL with the negative control solution was carried out (Figure S2.2.10).

Interestingly, after 3 days of incubation, the weight loss is similar or even slightly higher with 50 µL compared to 100 µL. These results tend to indicate that the amidase E4143 was not involved in the weight loss measured for the PCL-based TPU incubated with the enzymes mix. The amidase E4143 alone did not induce weight loss. The TPU structure and/or the polymer architecture might

make urethane bonds inaccessible to the amidase E4143 or this enzyme might hydrolyze urethane bonds without leading to detectable weight loss. Contrarily, the esterase E3576 was highly efficient for the depolymerization of PCL-based TPU.

Weight loss measurement is a good tool to appreciate the global efficiency of the degradation. However, some limitations can be noticed. For instance, degradation may be too superficial to be detected. It is then advised to perform polymer surface analysis. Moreover, release of molecular fragments that do not necessarily totally break down can be a main contributor to weight loss. Analysis of degradation products is thus also recommended.

TPU pieces and liquid fractions recovered after 51 days of incubation were subjected to further analysis. The physical modifications of TPU pieces were first visually evaluated. Observation of the whole PCL-based TPU pieces revealed rough, yellow surfaces for polymers incubated with the esterase E3576 (Figure 2.2.5a) and E4143/E3576 (Figure S2.2.11) while the PCL-based TPU incubated with the negative control and the amidase E4143 remained white and smooth. These observations were confirmed by SEM characterization showing that the yellow and rough aspect corresponds to deep cracks all over the piece (Figure 2.2.5b and c).

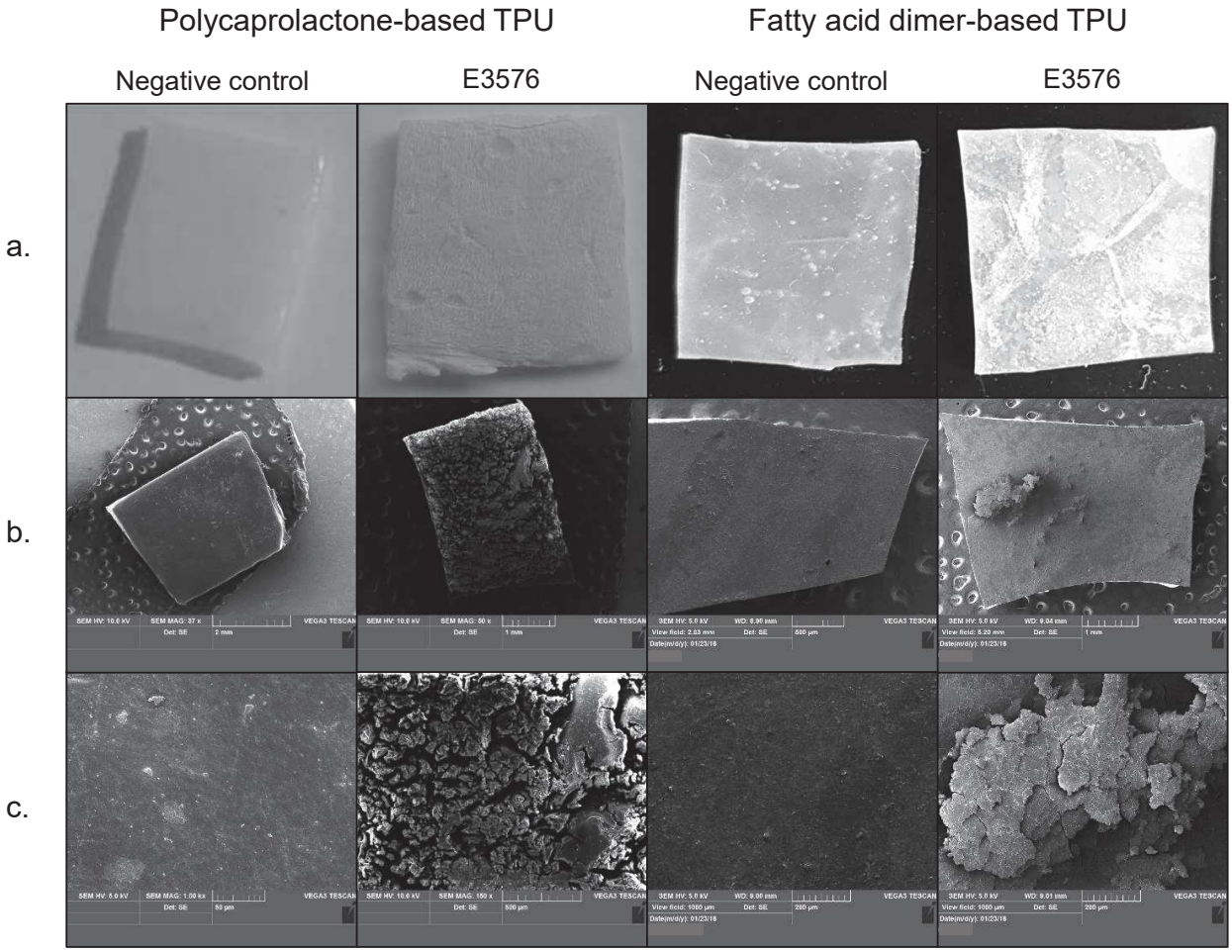


Figure 2.2.5 – Observation of degraded TPU pieces (a) naked eye aspect of the whole TPU (b) microscopic observation of the TPU pieces surface and (c) zoomed view on a degraded part of polymers

Fatty acid dimer-based TPU incubated with both the esterase E3576 (Figure 2.2.5a) and E4143/E3576 (Figure S2.2.11) presented dispersed whitish spots on the surface. SEM observations of TPU incubated with the esterase E3576 showed a degradation zone appearing as superimposed spots of polymers (Figure 2.2.5b and c).

For PCL-based TPU, the material initially present in the cracks is progressively released in the liquid fraction leading to important weight loss while for fatty acid dimer-based TPU no weight loss was observed, on agreement with SEM observation. Degradation seems to occur only at the top layer and no weight loss was observed for these pieces.

To complete the characterization of the degraded TPU pieces, SEC analysis were performed on the PCL- and the fatty acid dimer-based TPU. For this purpose, all remaining TPU incubated with the negative control or each of the enzymatic solutions were solubilized in THF. All the chromatograms presented a high intensity peak corresponding to the main polymer chains distribution (Figure 2.2.6). For the PCL-based TPU, a slight decrease of Mw and Đ were measured while Mn remained unchanged when incubated with the esterase E3576 and E4143/E3576 (Figure 2.2.6a and Table S2.2.3). No significant changes in the main polymer chain distribution were observed for the fatty acid dimer-based TPU (Figure 2.2.6b and Table S2.2.3).

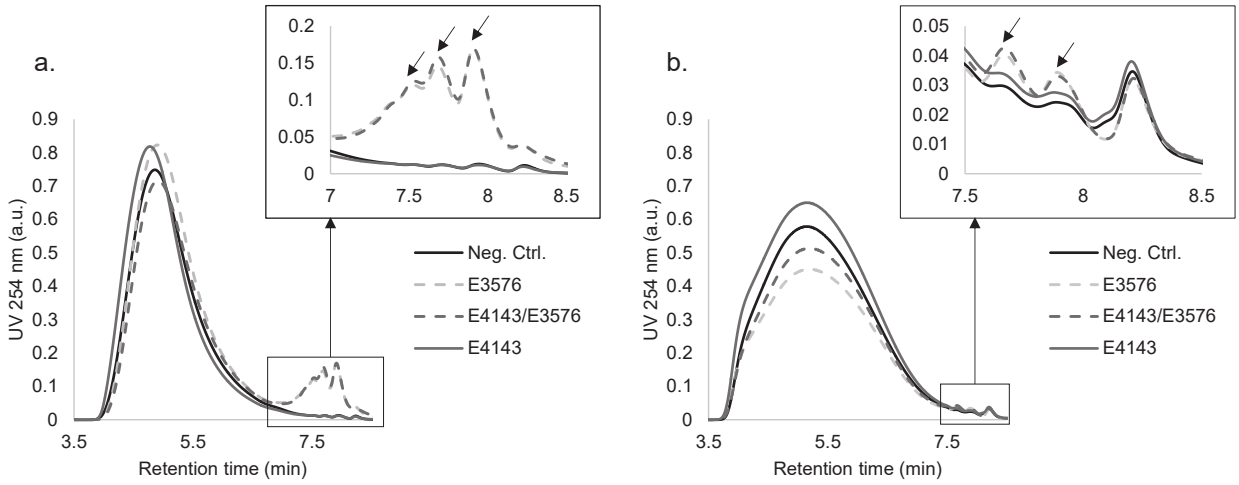


Figure 2.2.6 – SEC profiles of (a) the PCL-based TPU and (b) the fatty acid dimer-based TPU with zoomed views on the low molar mass oligomers populations.

However, for both TPU, the formation of low molar mass oligomers appeared after incubation with the esterase E3576 and E4143/E3576 mix. For the PCL-based TPU, four oligomer populations were clearly observed with Mw values between 500 and 3 000 g/mol, while two oligomer populations were detected for the fatty acid dimer-based TPU with Mw values between 1 500 and 2 500 g/mol. The appearance of such degradation products measured by SEC has already been described for bacterial degradation of a polyester, the poly(butylene adipate) (Trinh Tan et al. 2008).

The molar mass distributions of the PCL-based TPU vary only slightly and even no variations were observed for the fatty acid dimer-based TPU. The latter observation likely shows that degradation of these polymers mainly occurred at the surface of the pieces of TPU materials. The oligomers detected by SEC originate probably from the chains located at the surface area surrounding the cracks or at the superimposed patches observed by SEM. For the PCL-based TPU, SEM observations and SEC analyses clearly highlighted this surface degradation phenomenon. In addition to the weight loss already observed the remaining TPU pieces were highly deteriorated.

^{1}H-NMR was used to identify the main degradation products released under the action of esterase E3576 and similar analysis was performed on the negative control medium. The analysis of the degradation products present in the liquid fraction was conducted only for the PCL-based TPU since it is the only TPU leading to a significant weight loss. The cumulated PCL-based TPU weight loss of the three replicates for E3576 reactions was 6.6 mg. After the chloroform purification step, 2.2 mg of degradation products with a few possible salts and enzymes remnants were recovered. For the negative control, no polymer weight loss was detected during the degradation reaction and only 0.2 mg was recovered from the liquid fraction meaning that only few enzymes or salts might remain.

As E3576 is an esterase, a majority of caprolactone derivatives were expected to be found in the soluble fraction. Full NMR spectra are presented in supplementary materials (Figure S2.2.12). Two characteristic signals for PCL are usually used as reference peaks: (i) the methylene protons in alpha of the ester bond at about δ= 4.1 ppm and (ii) the methylene protons in alpha of the terminal hydroxyl group at about δ= 3.6 ppm (Duchiron et al. 2017). These two peaks were observed on the spectrum of the degradation products from E3576 but undetected for the negative control (Figure 2.2.7). The intensity of the signal at 3.6 ppm was nine-time higher than the signal at 4.1 ppm. Thus, more than 90% of the degradation products correspond to the constitutive monomer of the PCL, the 6-hydroxycaproic acid. This molecule has already been described as a degradation product of the PCL but, as far as we know, it is the first time for a PCL-based TPU (Tokiwa, Suzuki, and Takeda 1988). According to the signal intensities, the remaining degradation products (less than 10%) are thus composed of very short oligomers.

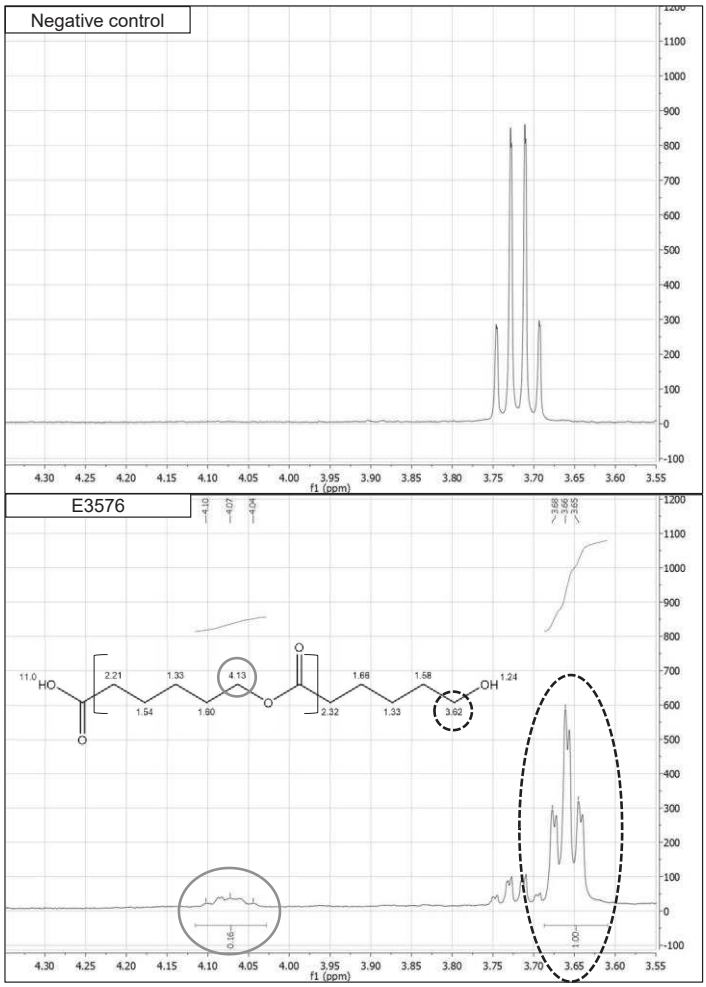


Figure 2.2.7 – NMR analysis of the caprolactone-derived degradation products resulting from 3 days hydrolysis of the PCL-based TPU with the negative control (top) and the esterase E3576 (bottom)

Another analytical method was used for the degradation products analysis to better understand the depolymerization mechanism of the urethane bond hydrolysis. One of the degradation products of 4,4'-MDI-based TPU is the 4,4'methylenedianiline (MDA), a bicyclic diamine (Tang, Labow, and Santerre 2003b). The method of detection of MDA and MDA-derivatives by LC/ESI/qTOF-MS, previously described by Gamerith *et al.* (Gamerith et al. 2016), was thus employed. This technique allows the detection of extremely low MDA concentration (10 mg/L) (Figure S2.2.13). In MS chromatograms, MDA peaks of samples and standard range solutions were within the background noise for concentrations lower than 2 mg/L. To detect it, ion extraction was required as performed in the reference publication (Gamerith et al. 2016).

Contrary to the previous NMR analysis, LC/ESI/qTOF-MS analyses were performed on the soluble fractions recovered all along the 51 days of the assays with the esterase E3576, the amidase E4143 and the E4143/E3576 mix. The soluble fractions of each degradation assay recovered every 3 or 4 days were pooled together to analyze the products released along the whole reaction time. MDA was not detected in the negative control. Concerning E3576 it was detected in only one of the replicate but in very low concentration (Figure 2.2.8a). In contrast, MDA was detected in the liquid fractions of all the replicates of reactions involving E4143 and E4143/3576 mix and the measured concentrations were 0.35 and 0.25 mg/L, respectively. Interestingly, an MDA derivative corresponding to an MDA linked to a caprolactone unit by a urethane bond was only detected in the liquid fraction of TPU incubated with E4143/E3576 (Figure 2.2.8b). The concentration of this molecule was 2.9 mg/L, which is ten-time higher than the MDA concentration in the same samples.

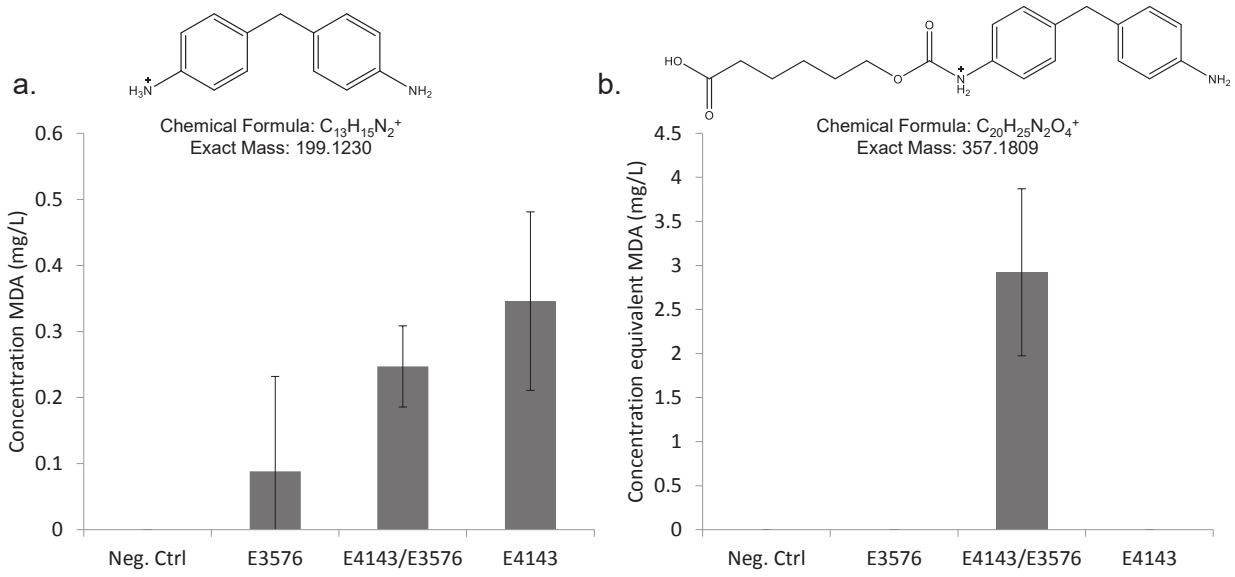


Figure 2.2.8 - Measurement of the concentration of degradation products in the liquid fractions of enzymatic reaction. (a) 4,4'-methylenedianiline and (b) 4,4'-methylene dianiline linked to a caprolactone by an urethane bond

According to these results, one may postulate that esterase E3576 first degrades the macromolecules and releases in the supernatant some smaller water soluble molecules containing urethane bond. It is then easier for the amidase E4143 to hydrolyze the urethane bond

of such low molar mass molecules. The degradation would thus occur in discrete steps. Similarly, Ozsagiroglu et al., (Ozsagiroglu, Iyisan, and Guvenilir 2012) suggested to use an enzymatic cocktail with an esterase and a protease to improve the hydrolysis of PU. However, they reported that enzymes compete with each other in cocktails and therefore do not cause any improvement in the depolymerization. Unlike theirs, the results of our experiments show that there was no competition but rather a synergistic action leading to the release of new specific building blocks. However, the performed measurements showed that this synergistic action did not lead to enhanced weight loss.

It seems that the molecule with MDA linked to a caprolactone unit accumulates in the medium since the corresponding urethane bond of this molecule was not cleaved. A similar case had already been described for the PET enzymatic hydrolysis with the cutinase TfCut2 from *Thermobifida fusca* (Barth et al. 2016). TfCut2 was able to cleave one ester bond thus releasing an ethylene glycol and a mono-(2-hydroxyethyl-) terephthalate which is an inhibitor of TfCut2. In our case, a similar mechanism might be involved. E4143 is probably able to cleave a urethane bond releasing a 6-hydroxycaproic acid and a potential inhibitor still containing a urethane bond. Improvement of the process to limit the inhibitory impact could lead to higher degradation activity for the amidase E4143. For example, an integrated process using an ultrafiltration membrane reactor may allow the continuous removal of the inhibitor while conserving the enzymatic activity (Barth et al. 2015).

Three main degradation products were identified in these experiments, the 6-hydroxycaproic acid, the MDA and the MDA linked to a caprolactone unit. The first one was recovered at a concentration of about 1 g/L and the two others were detected at concentrations ranging from 0.3-3 mg/L.

The efficiency of this system on a PCL-based TPU is mainly due to the PCL segment of the polymer which is known to be biodegradable (Daemi et al. 2016). Applicability toward other types of PU still needs to be demonstrated. However, the identification of degradation products resulting from urethane bond hydrolysis tends to show that this technique can be improved and adapted to other types of PU. Improvement may concern the surface of interaction between the polymer

and the enzyme, the conditions of reaction (pH, shaking and temperature) but also enzymes engineering which may lead to enhanced enzymatic activities.

5. Conclusion

This study shows the successful implementation of two enzymatic screenings addressing poly(ester urethane) degradation and specific hydrolytic activity on the urethane bond. The activity of the two enzymes selected from each screening was then evaluated on TPU. The esterase E3576 reveals a high degradation activity on a poly(ester urethane) based on PCL and a weak degradation activity on the top layer of a poly(ester urethane) based on fatty acid dimer. Combining the esterase E3576 and the amidase E4143 led to an increase in urethane bond hydrolysis compared to degradation reactions with each enzyme alone.

Most studies on the enzymatic depolymerization of PU focused only on the ester part of the polymers. This paper goes one step further by offering a new enzymatic system for the efficient depolymerization of the TPU soft segment (through the ester bonds hydrolysis) while also tackling the hard segment (via the urethane bond cleavage) for a full degradation of PU.

Interestingly, the identified degradation products could be recovered and valorized as building blocks for the synthesis of a second generation of polymers. For instance, by polycondensation, new polyesters can be synthesized from the carboxylic acids and alcohols (Debuissy et al. 2017) while polyamides could be obtained from the recovered amines building blocks (Hablot et al. 2010; Li et al. 2002).

The general approach presented in this paper is focused on bio-recycling systems based on a sustainable “Biotech-Chem” cycle. This cycle, based on first a biotech step, with a controlled biodegradation to obtain building blocks, followed by a chemical synthesis step to form new macromolecular architectures, could be applied on a great number of potentially cleavable polymers, mainly obtained by polyaddition or polycondensation.

Acknowledgments

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7. Supporting information

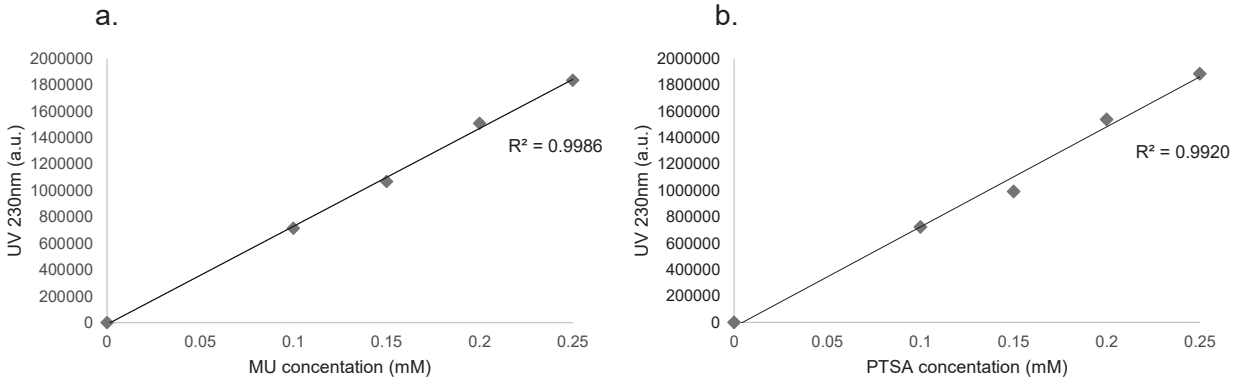

Figure S2.2.9 – Calibration curves of (a) the mono-urethane and (b) the p-Toluenesulfonamide performed by HPLC with absorbance measurement at 230 nm

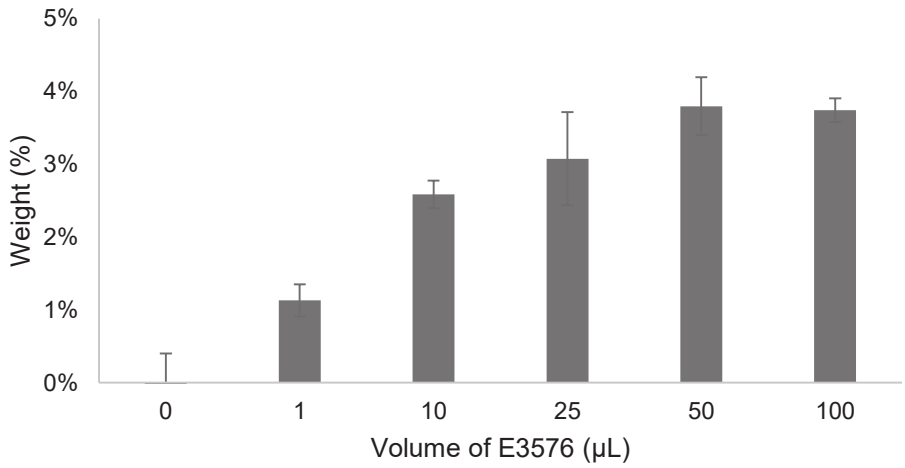

Figure S2.2.10 – Evaluation of the weight loss for PCL-based TPU after 3 days of incubation with different volume of E3576

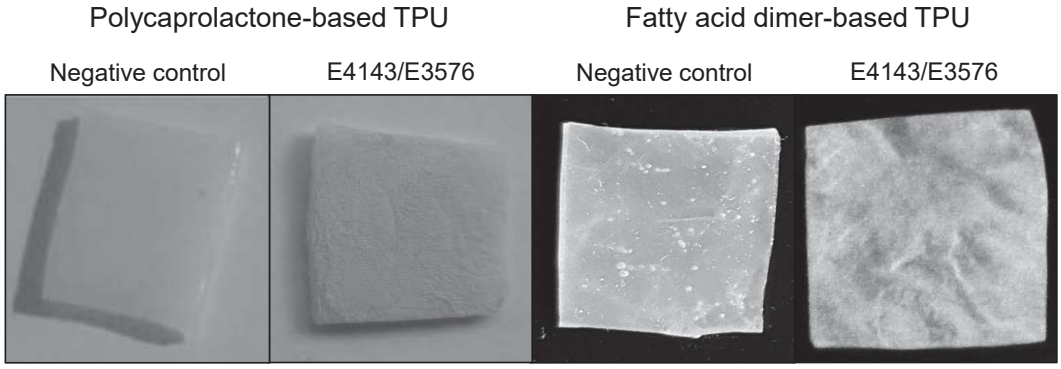

Figure S2.2.11 – Observation of degraded TPU pieces incubated 51days at 37°C with the mix of amidase and esterase E4143/E3576: naked eye aspect of the whole TPU

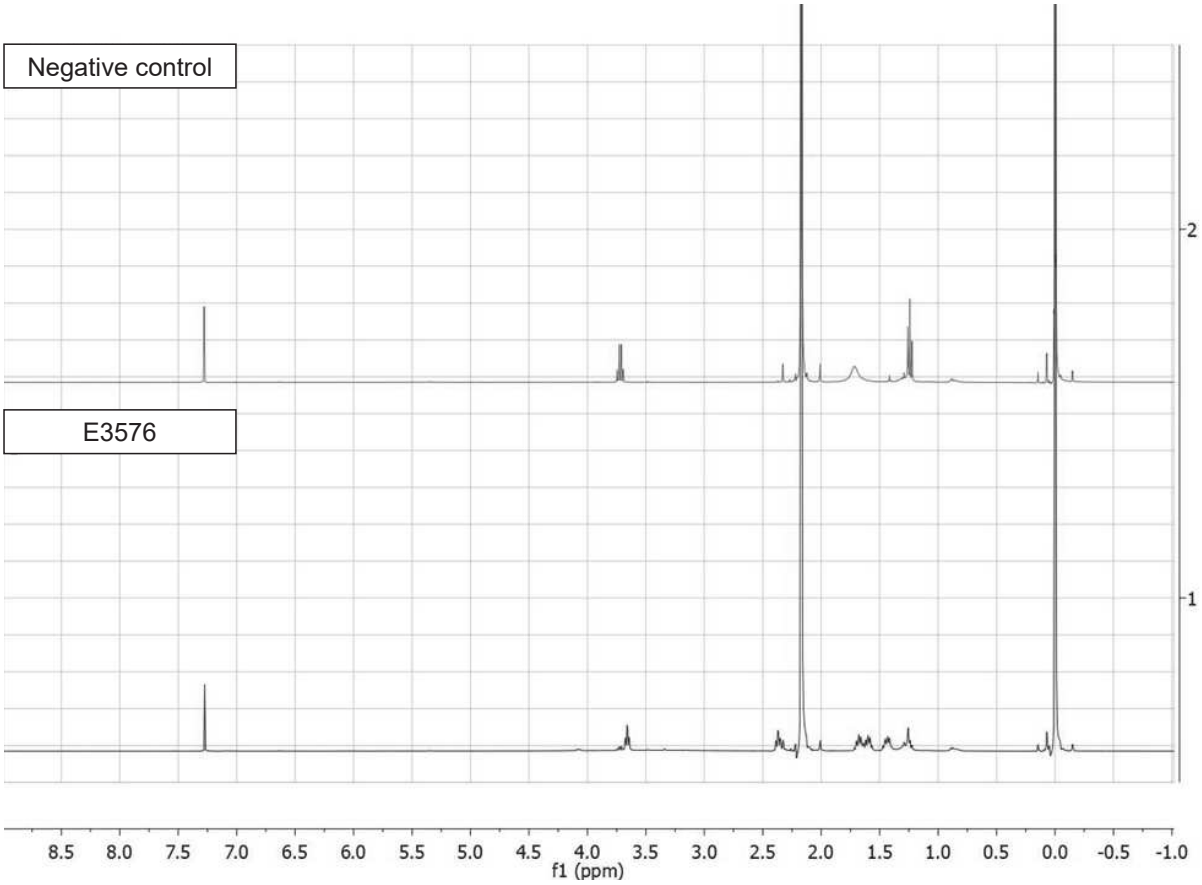

Figure S2.2.12 – Full NMR spectra of the degradation products after 3 days of incubation of the PCL-based TPU with the negative control and the esterase E3576

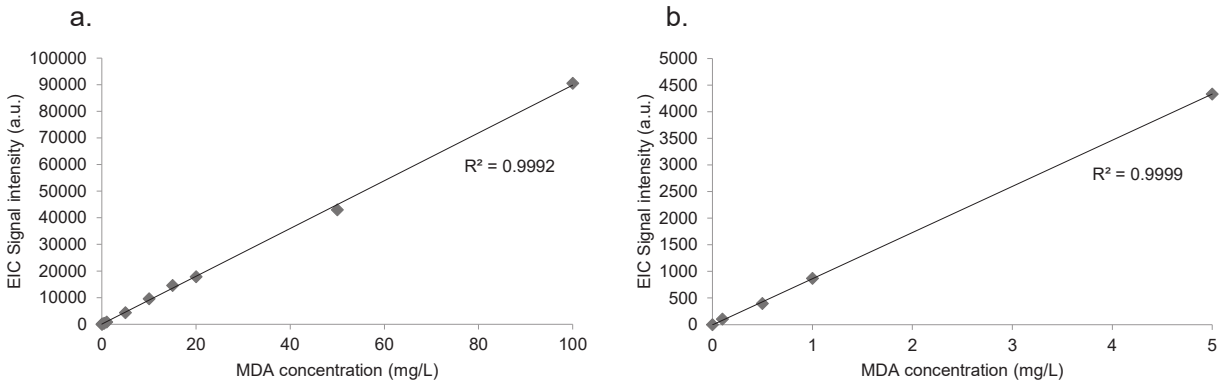

Figure S2.2.13 – Calibration curve of 4,4'-MDA (a) between 100 and 0.1 mg/mL and (b) between 5 and 0.1 mg/L performed by LC/ESI/qTOF-MS. Data obtained from Extracted Ion Chromatogram. (EIC)

Table S2.2.2 – SEC results in tetrahydrofuran. Average molar masses and dispersity of the different synthesized TPU

	PCL based TPU	Fatty acid dimer based TPU	PTHF based TPU	Poly(ester ether) based TPU
Mn (kg/mol)	54.1	35.2	56.0	34.7
Mw (kg/mol)	115.7	120.2	162.7	75.1
Đ	2.14	3.41	2.91	2.17

Table S2.2.3 – SEC results in tetrahydrofuran. Average molar masses and dispersity of the PCL-based and fatty acid dimer-based TPU incubated with the negative control, the esterase E3576, the amidase E4143 and the mix E4143/E3576

			Neg. Ctrl.	E3576	E4143/ E3576	E4143
PCL-based TPU	Mn (kg/mol)	Mean	57.0	58.6	61.3	60.3
		Std. Dev.	1.4	3.2	5.1	3.7
	Mw (kg/mol)	Mean	120.7	105.0	110.3	130.2
		Std. Dev.	3.6	5.0	1.0	10.1
	Đ	Mean	2.12	1.79	1.80	2.16
		Std. Dev.	0.01	0.03	0.03	0.04
Fatty acid dimer-based TPU	Mn (kg/mol)	Mean	34.6	36.0	35.9	34.7
		Std. Dev.	0.3	0.7	0.3	0.4
	Mw (kg/mol)	Mean	128.3	133.5	129.4	135.9
		Std. Dev.	5.9	4.2	5.9	3.2
	Đ	Mean	3.71	3.70	3.61	3.91
		Std. Dev.	0.20	0.05	0.14	0.05

Sous-chapitre 2.3. Expériences complémentaires

Pour compléter et approfondir les travaux précédents, la stratégie prévue est d'améliorer les compétences de dégradation de l'enzyme E4143. En effet, les sous-chapitres précédents ont démontré son activité sur des uréthanes de faibles masses molaires mais une activité négligeable sur des PU. L'ajout d'un domaine de liaison (Surface-Binding Domain ou SBD) a donc été envisagé. Cette méthode consiste à ajouter une séquence d'acides aminés à la séquence peptidique de l'enzyme d'intérêt pour permettre l'ancrage de l'enzyme par adsorption à la surface du polymère grâce à une queue hydrophobe. L'ajout d'un domaine de liaison a déjà montré son efficacité sur des enzymes hydrolysant le PET (Ribitsch et al. 2015; Espino-Rammer et al. 2013; Perz et al. 2015).

L'ajout du SBD fut suivi d'une phase d'évolution dirigée visant à améliorer l'activité de clivage de la liaison uréthane. L'évolution dirigée est une technique permettant d'améliorer les propriétés d'une enzyme par introduction de mutations (Zeymer and Hilvert 2018). Ces mutations sont induites aléatoirement au sein d'une séquence peptidique pour générer une collection de variants. Par la suite, un criblage à haut débit de cette collection est réalisé pour sélectionner les enzymes modifiées présentant, une amélioration de l'activité d'hydrolyse de la liaison uréthane. Ce criblage est réalisé en microplaque sur une molécule modèle de faible masse molaire contenant deux liaisons uréthanes.

A l'issu des modifications de l'enzyme E4143 et du criblage, deux mutants ont présenté une augmentation de l'activité hydrolytique sur la liaison uréthane : 78H4 et 79A11. Ces mutants ont fait l'objet d'une caractérisation plus approfondie au sein de notre laboratoire.

L'activité des mutants a été testée sur des molécules modèles : le pNitrophenyl acétate (pNPA) et le pNitroacetaniline (pNAA) (Figure 2.3.1a et b), comparativement à l'enzyme native E4143. Ces substrats sont utilisés respectivement pour l'évaluation de l'activité estérase et amidase. La mesure de l'absorbance à 405 nm permet de suivre l'hydrolyse des liaisons et donc de révéler et quantifier l'activité enzymatique. La concentration en protéines a été mesurée par un test de Bradford pour exprimer l'activité en fonction de la quantité de protéines. L'activité enzymatique est donc exprimée en µmol de pNitrophénol ou pNitroaniline/min.mg de protéines. Les mesures

effectuées sur ces deux molécules modèles ont montré que les activités estérase et amidase apparaissent augmentées de l'ordre d'un facteur 5 environ pour les deux mutants en comparaison de l'enzyme native (Figure 2.3.1c).

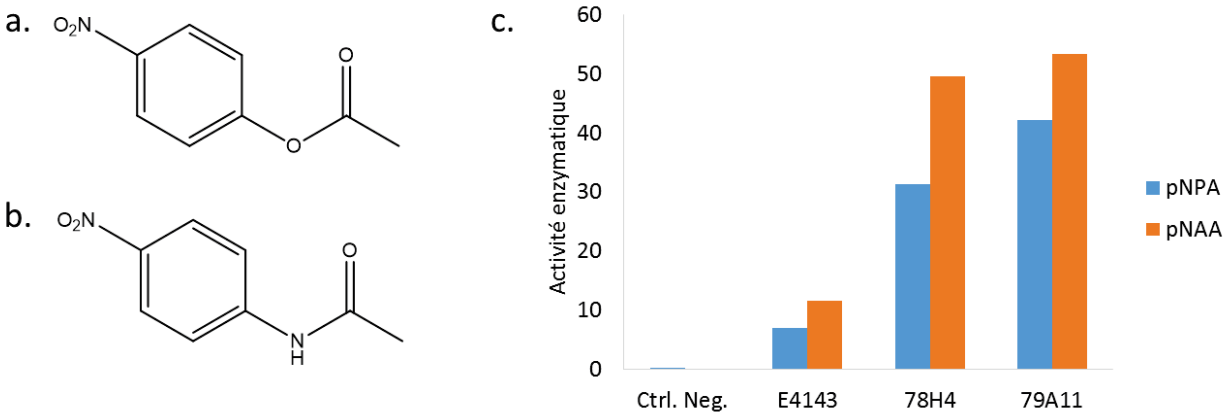

Figure 2.3.1 – Molécules modèles pour l'évaluation de l'activité enzymatique: (a) pNitrophenyl acétate and (b) pNitroacetanilide. (c) Activités esterase et amidase avec E4143, 78H4 et 79A11

Considérant l'ajout du SBD et la capacité augmentée des mutants à hydrolyser la liaison ester présentée ci-dessus, nous avons souhaité évaluer l'activité des mutants sur l'Impranil-DLN®. Pour rappel, l'Impranil-DLN® est un polyester PU en dispersion aqueuse. Il est d'aspect laiteux mais devient transparent lors de sa dégradation ce qui permet un suivi par mesure d'absorbance à 600 nm. Il a été démontré lors du criblage dans le sous-chapitre précédent que l'enzyme native n'avait pas d'activité sur l'Impranil-DLN®. L'enzyme E3576, qui présente une activité estérase, a été utilisée comme contrôle positif diluée au dixième dans cette expérience. Aucune baisse d'absorbance n'est mesurée pour les mutants 78H4 et 79A11 (Figure 2.3.2 – Evolution de l'absorbance à 600 nm de l'Impranil-DLN incubé avec E3576, 78H4 et 79A11. L'adjonction d'un domaine d'ancrage des enzymes sur le substrat et l'augmentation de l'activité catalytique ne sont donc pas suffisants pour hydrolyser l'Impranil-DLN®.

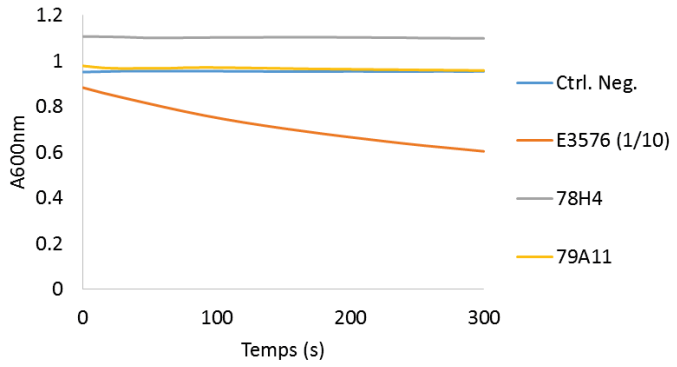

Figure 2.3.2 – Evolution de l'absorbance à 600 nm de l'Impranil-DLN incubé avec E3576, 78H4 et 79A11

Nous avons ensuite testé l'activité des mutants sur des polyesters TPU (Figure 2.3.3). Deux polymères ont été utilisés : le TPU base PCL et le TPU base dimères d'acides gras. En effet, seul ces deux polymères ont montré une sensibilité à la dégradation enzymatique dans le sous-chapitre précédent. Les conditions expérimentales ont été décrites auparavant (Sous-chapitre 2.2.3.3.3 TPU degradation assay, page 100). En plus d'évaluer l'activité des mutants seuls, un mélange contenant un volume équivalent de 78H4 ou 79A11 et d'E3576 a été testé. Aucune perte de masse n'a été relevée pour les TPU base PCL incubés avec 78H4 et 79A11 (Figure 2.3.3a). Les pertes de masses mesurées pour 78H4-E3576 et 79A11-E3576 sont similaires à celles mesurées pour E3576 et pour E4143-E3576. Aucune enzyme ou mix d'enzymes n'a entrainé de perte de masse sur le TPU base dimère d'acide gras (Figure 2.3.3). Les performances des mutants étant similaires à celles de l'enzyme native E4143, aussi bien seules qu'en mélange avec E3576, nous pouvons donc en conclure que les modifications apportées à l'enzyme n'entrainent pas d'amélioration d'activité pour la dégradation des polyesters TPU.

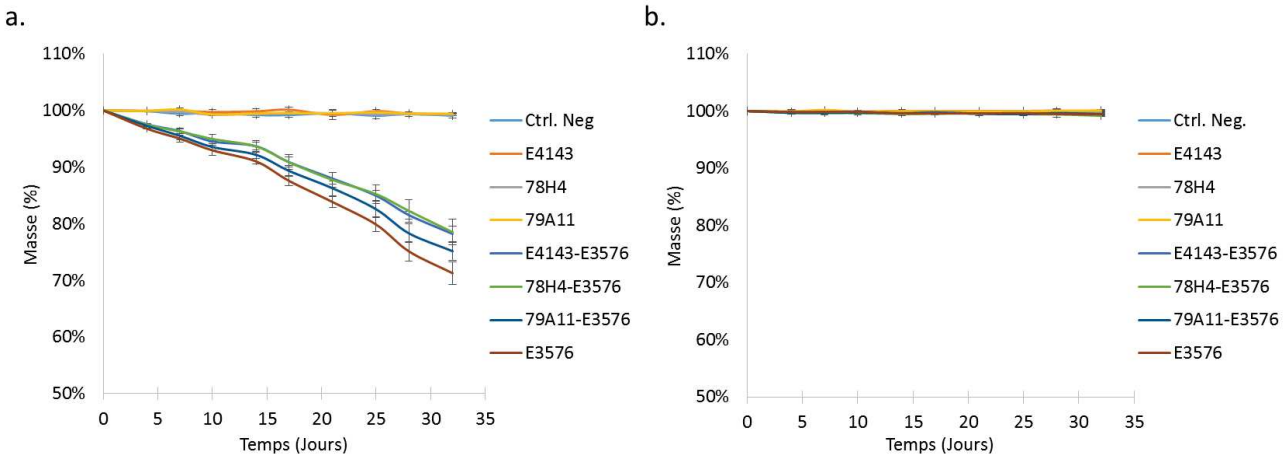

Figure 2.3.3 – Evolution de la masse du (a) TPU à base de PCL et du (b) TPU à base de dimères d'acides gras incubés avec différentes enzymes

Dans ce sous-chapitre, les essais sur substrats modèles ont montré qu'en plus d'une augmentation de l'activité hydrolytique sur la liaison uréthane, les mutants présentent également une activité hydrolytique accrue sur les liaisons esters et amides par rapport à l'enzyme native E4143. Cependant les modifications apportées à l'enzyme ne sont pas suffisantes pour hydrolyser l'Impranil-DLN, un polyester PU en dispersion aqueuse. Finalement, les modifications subies par E4143 ne permettent pas d'améliorer son activité de dégradation des polyesters TPU.

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Ribitsch, D., E. Herrero Acero, A. Przylucka, S. Zitzenbacher, A. Marold, C. Gamerith, R. Tscheliessnig, A. Jungbauer, H. Rennhofer, H. Lichtenegger, H. Amenitsch, K. Bonazza, C. P. Kubicek, I. S. Druzhinina, and G. M. Guebitz. 2015. 'Enhanced cutinase-catalyzed hydrolysis of polyethylene terephthalate by covalent fusion to hydrophobins', *Appl Environ Microbiol*, 81: 3586-92.

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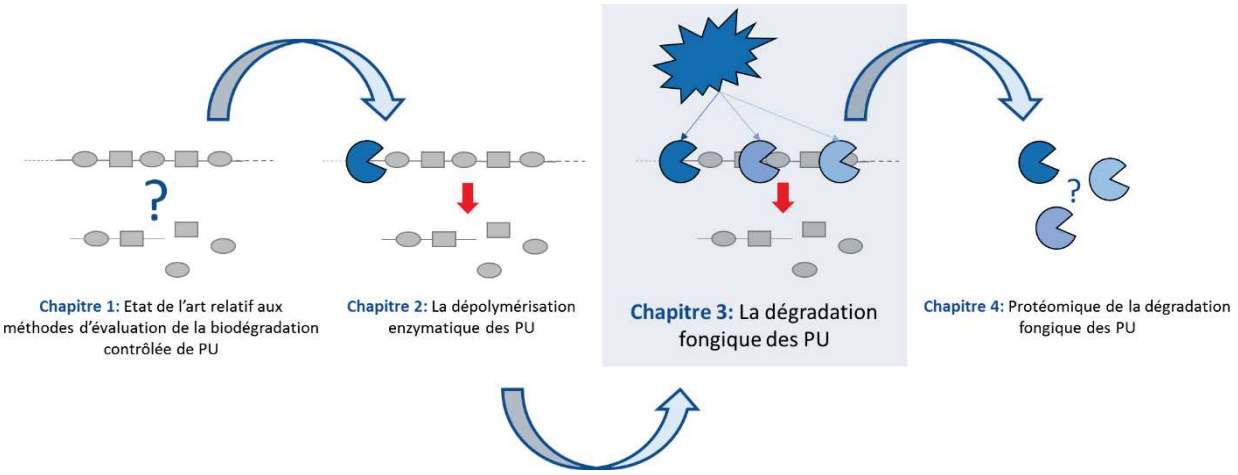
Conclusion

Cette partie avait pour objectif l'identification et la caractérisation d'enzymes de dégradation des polyuréthanes à partir d'une collection de 50 hydrolases. Une série d'expériences préliminaires a souligné la pertinence de l'utilisation de substrats modèles pour cribler simultanément et efficacement une collection d'enzymes.

Les criblages ont révélé 4 enzymes présentant une activité de dégradation sur un polyester PU en dispersion aqueuse et 2 enzymes ayant une activité de dégradation sur un substrat uréthane de faible masse molaire. Parmi ces enzymes, une estérase, E3576 et une amidase, E4143, ont été sélectionnées pour des tests de dégradation sur des substrats plus complexes et réalistes que sont les TPU. L'enzyme E3576 a ainsi engendré 33% de perte de masse après 51 jours d'incubation à 37°C sur un TPU base PCL et une légère dégradation de surface sur un TPU base dimères d'acides gras dans les mêmes conditions. L'activité de l'enzyme E4143 s'est avérée négligeable sur les TPU. Cependant, le relargage en quantité significative d'un produit de dégradation a révélé une synergie entre les enzymes E4143 et E3576 en mélange. L'estérase clive les fonctions esters des TPU libérant ainsi des fragments plus courts pouvant être hydrolysé au niveau des liaisons uréthanes par E4143.

La modification de l'enzyme E4143 par ajout d'un domaine de liaison et évolution dirigée a fourni deux mutants présentant une activité hydrolytique améliorée sur la liaison uréthane. Des essais complémentaires ont montré que, comparativement à l'enzyme native, l'activité hydrolytique des mutants était également meilleure sur les liaisons amides et esters de molécules modèles. En revanche, cette amélioration de l'activité s'est avérée insuffisante pour pouvoir hydrolyser significativement des substrats du type TPU à base de polyesters.

Chapitre 3. La dégradation fongique des polyuréthanes



Introduction

Comme nous l'avons vu dans le chapitre précédent, l'utilisation d'enzymes, seules ou en mélange, s'est révélée efficace pour la dégradation de substrats uréthanes modèles et de polyesters TPU. Dans la continuité de ces travaux, nous avons souhaité nous intéresser à la dégradation des PU par voie microbienne. Comme présenté dans le premier chapitre, de nombreuses publications soulignent la capacité de dégradation élevée des champignons. Une collection de souches de champignons susceptibles d'avoir une activité de dégradation sur les PU a donc été établie.

La sélection de champignons d'intérêt a nécessité la mise au point de criblages. Ceci fait l'objet d'une première sous-partie. Contrairement aux expériences sur les enzymes, il a été possible de travailler directement sur des TPU. La réalisation des criblages ainsi que la caractérisation des champignons et de leurs compétences de dégradation sont ensuite présentées sous forme d'une publication scientifique qui constitue le cœur de ce chapitre. Finalement, des expériences complémentaires ont été rapportées, ceci afin d'identifier les produits de dégradation et ainsi tenter d'élucider les mécanismes impliqués.

Sous-chapitre 3.1. Développement de criblages fongiques

La première étape de ce développement de criblage fut la collecte de souches de champignons d'intérêts. Cette étape est détaillée dans la partie « matériel et méthode » du sous chapitre suivant (Sous-chapitre 3.2.3.2.1 Fungal isolation, page 142). Des échantillons ont été collectés dans différents sites où ils étaient soumis à une exposition prolongée au contact de PU, puis déposés sur différents milieux de culture. Les souches de champignons présentes dans les échantillons ont été ensuite isolées par repiquages successifs sur milieux gélosés. La collection de champignon ainsi constituée est composée de 3 souches de levures et 24 souches de champignons filamenteux.

Afin de sélectionner les souches capables de dégrader les PU au sein de la collection établie, le développement d'un criblage a été nécessaire. Comme exposé précédemment, un des freins au développement de criblages enzymatiques est la dénaturation des enzymes après quelques jours de réaction. En revanche, les réactions de dégradation mettant en jeu des microorganismes peuvent être réalisées sur plusieurs mois. Il est possible de cribler les souches directement sur des TPU réels et complexes sans passer au préalable par des substrats modèles du type Impranil®, comme dans le chapitre précédent. Les conditions de culture (temps d'incubation, température, agitation) et le milieu de culture sont les paramètres à optimiser et/ou valider dans cette phase de développement.

Le premier essai de dégradation a été réalisé sur le polyester TPU base dimère d'acide gras décrit dans le chapitre précédent (Chapitre 2, Sous-chapitre 2). Pour cela, 10 billes de TPU (environ 200 mg) et 10 mL de Potatoes Dextrose Broth (PDB, utilisé selon les recommandations du fournisseur, à savoir 24 g/L) ou de milieu minimum (MM, dont la composition est détaillée dans le matériel et méthode du sous chapitre suivant) ont été disposés dans des flacons de 50 mL. Le PDB est un milieu riche en nutriments complexes alors que le MM contient des minéraux, de l'azote mais est dépourvu de carbone. Cinq souches de la collection ont été sélectionnées aléatoirement pour être testées au cours de cette expérience préliminaire. L'incubation a été réalisée à 30°C, sous agitation orbitale à 120 rpm. Afin d'évaluer la biodégradation, deux

techniques différentes ont été choisies : la perte de masse de l'échantillon et la diminution de la masse molaire du polymère mesurée par chromatographie d'exclusion stérique (SEC).

Après deux semaines d'incubation dans le PDB, une croissance significative a été observée pour les 5 souches. La croissance des champignons ne semblant plus évoluer après ces deux semaines, les réactions ont été stoppées. La perte de masse a été évaluée sur la masse totale des 10 billes et deux billes de chaque réaction ont été analysées par SEC. Aucune perte de masse et aucune baisse de la masse molaire moyenne en masse (Mw) n'ont été mesurées (Figure 3.1.1).

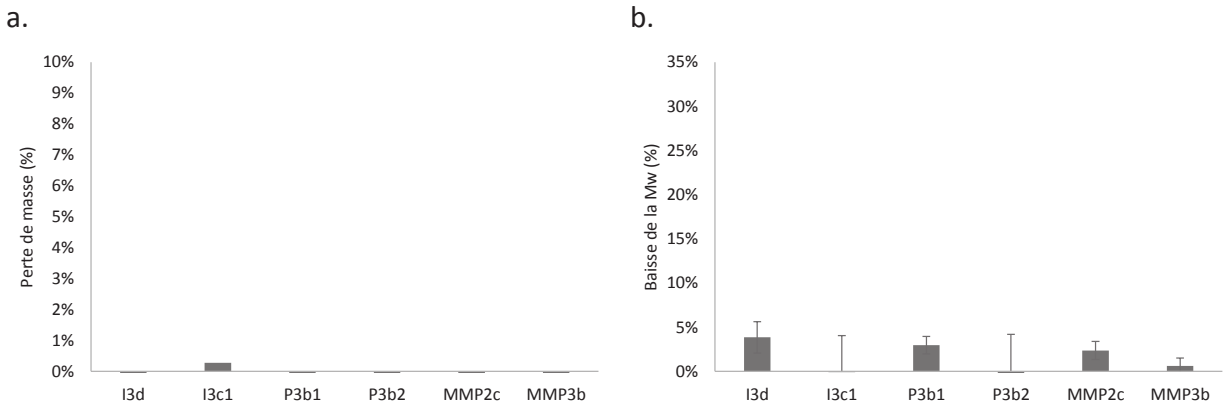


Figure 3.1.1 – Analyse des TPU base dimère d'acide gras incubé deux semaines à 30°C dans du PDB par (a) perte de masse et (b) baisse de la Mw

Après deux semaines d'incubation, aucune croissance n'a été observée pour les cinq souches incubées en MM avec les billes de TPU. Le MM étant dépourvu de carbone, l'absence de croissance révèle que ces champignons ne peuvent utiliser le TPU comme source de carbone et donc l'absence de dégradation. Toutefois, après 6 semaines d'incubation, la formation d'un mycélium conséquent autour des billes de TPU incubées avec les souches I3c1 et MMP3b a été observée (Figure 3.1.2a). Les réactions ont alors été arrêtées. Pour les polymères incubés avec ces deux souches, une perte de masse de l'ordre de 2% a été mesurée, accompagnée d'une diminution de masse molaire de l'ordre de 25% (Figure 3.1.2b et c).

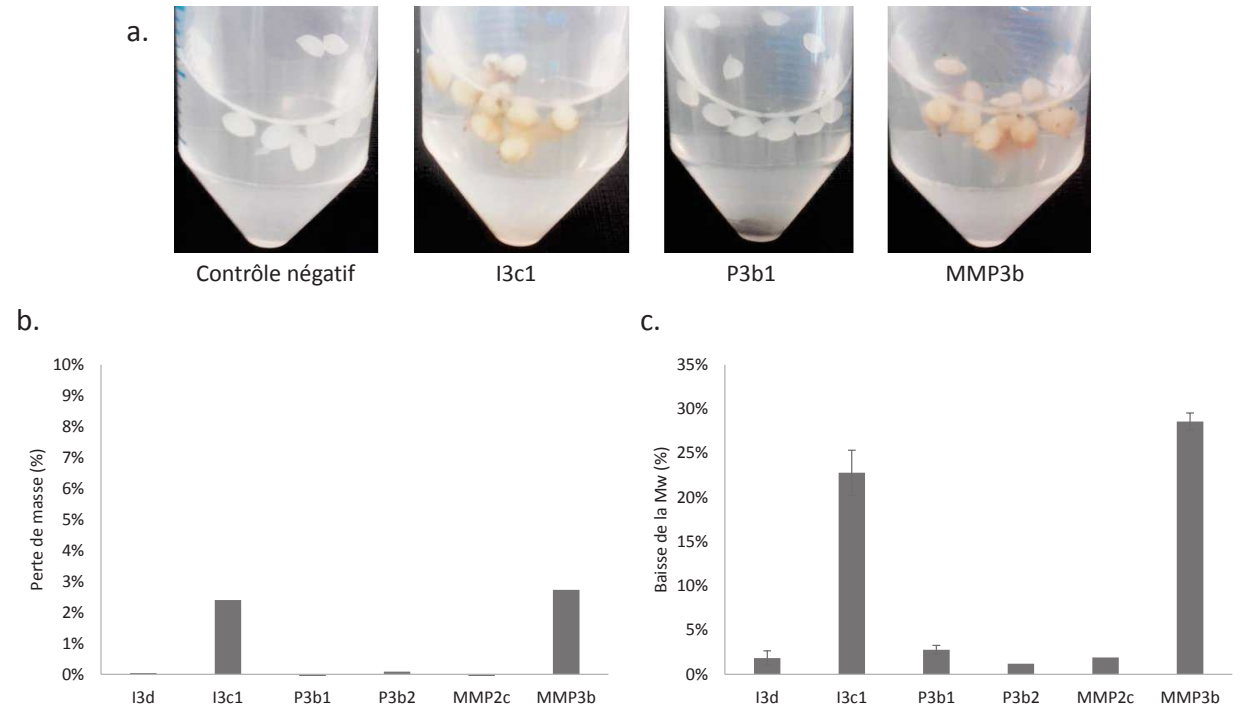


Figure 3.1.2 – Analyse des TPU base dimère d'acide gras incubés deux semaines à 30°C dans du MM par (a) évolution de la croissance fongique, (b) perte de masse et (c) baisse de la Mw

Les souches I3c1 et MMP3b ont montré une activité sur le TPU lors de l'incubation avec le MM mais pas avec le PDB. Cela montre que l'accès à des substrats facilement assimilables présents dans ce milieu riche semble être un frein à la production d'enzymes de dégradation. En revanche, l'incubation en MM contraint les champignons à produire un arsenal enzymatique qui semble efficace pour la dégradation des PU. Les incubations en PDB et MM n'ayant pas eu la même durée (2 et 6 semaines respectivement), il est également possible que cette différence soit la cause de l'absence de dégradation avec le PDB.

La première expérience préliminaire ayant été réalisée sur un nombre restreint de souches et avec des temps d'incubation différents, nous avons souhaité reproduire cette expérience avec l'ensemble de la collection de souches. Les conditions de réactions ont été optimisées afin de maximiser les interactions polymères/champignons. En effet, nous avons observé une sédimentation du champignon utilisé pour inoculer dans le cas de l'incubation du TPU en MM avec P3b1 (Figure 3.1.2a) limitant ces interactions. Aussi, les expériences sur l'intégralité des

souches ont été menées par des incubations de deux mois à 30°C sans agitation pour lesquelles un volume de 2 mL de PDB ou de MM a été ajouté à 5 billes de TPU dans un flacon de 40 mL. Ces conditions permettent une surface de contact optimisée par rapport à la première expérience.

Les pertes de masses mesurées pour le TPU incubé avec du PDB n'excèdent pas 1% alors que de nombreuses souches de la collection entraînent une perte de masse supérieure à 1% lors de l'incubation en MM (Figure 3.1.3). Cette expérience confirme que le MM est plus adapté que le PDB pour la dégradation du TPU et ceci indépendamment du temps d'incubation.

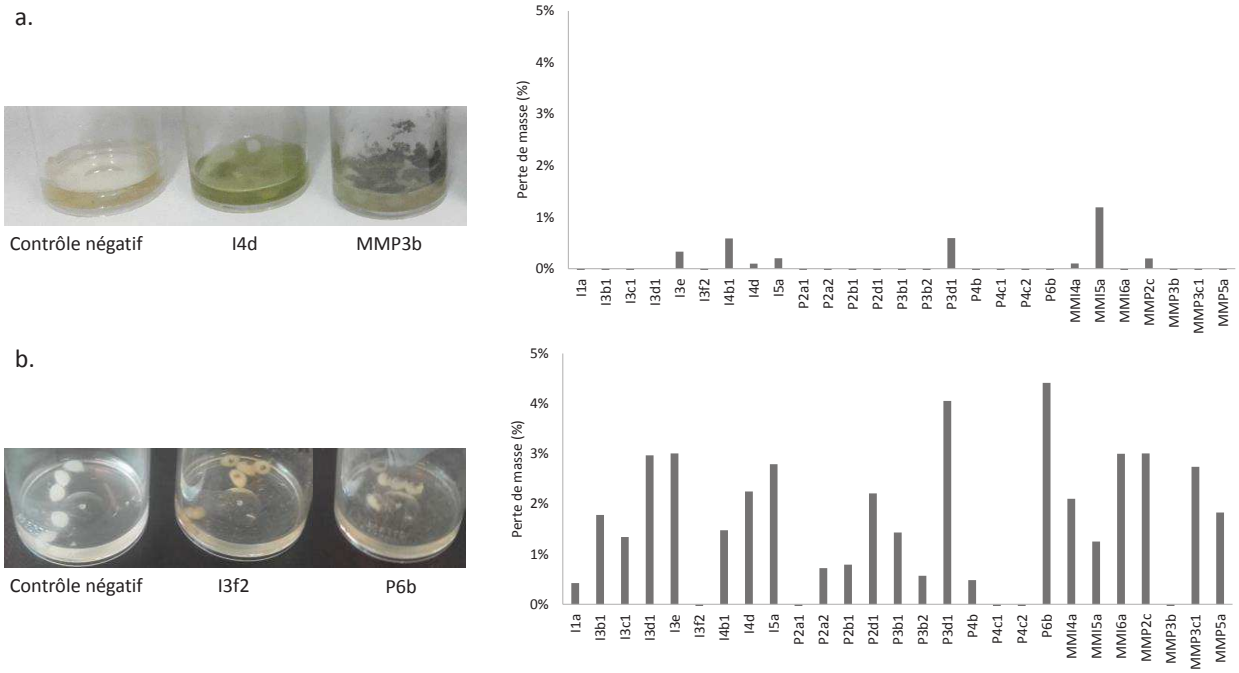


Figure 3.1.3 – Criblage de la collection de souches de champignons sur les billes de TPU (a) incubées avec du PDB et (b) incubées avec du MM

Cette série d'expériences montre qu'il est donc possible de réaliser un criblage en MM dans des flacons de 40 mL. Les conditions sélectionnées sont une incubation de deux mois sans agitation à 30°C. Ce criblage est facilement transposable à tous types de TPU. C'est ce qui est réalisé dans le sous-chapitre suivant.

Sous-chapitre 3.2. Isolation and characterization of different promising fungi for biological waste management of polyurethanes

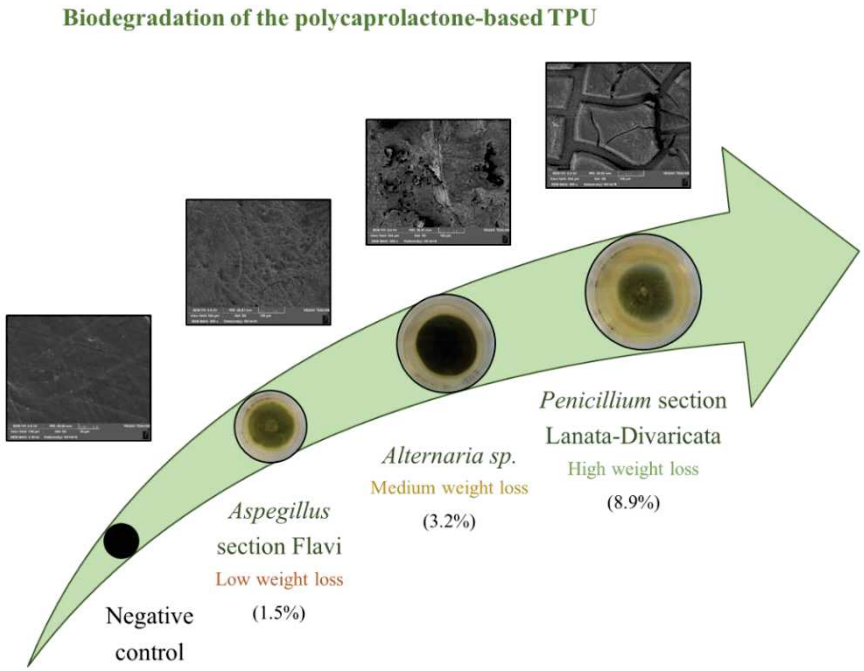
Audrey Magnin[1], Lucie Hoornaert[1], Eric Pollet[1], Stéphanie Laurichesse[2], Vincent Phalip[3], Luc Avérous[*,1]

(1) BioTeam/ICPEES-ECPM, UMR CNRS 7515, Université de Strasbourg, 25 rue Becquerel, 67087 Strasbourg Cedex 2, France

(2) Soprema, 14 rue de Saint-Nazaire, 67025 Strasbourg, Cedex 1, France

(3) Univ. Lille, INRA, ISA, Univ. Artois, Univ. Littoral Côte d'Opale, EA 7394 - ICV - Institut Charles Viollette, 59000 Lille, France

(*) Corresponding author: luc.averous@unistra.fr



1. Abstract

As a highly resistant polymer family, polyurethanes (PU) are responsible for increasing environmental issues. Then, PU biodegradation is a challenging way to develop sustainable waste management processes based on biological recycling. Since the metabolic diversity of fungi is a major asset for polymer degradation, nearly thirty strains were isolated from sampling on six different PU wastes-containing environments. A screening of the fungi on four thermoplastic PU (TPU) with different macromolecular architectures led to the selection of three strains able to use two polyester PU as sole carbon source: *Alternaria sp*., *Penicilium* section Lanata-Divaricata and *Aspergillus* section flavi. Weight loss, FT-IR, Scanning Electron Microscopy and Size Exclusion Chromatography analyses revealed that these three fungi degrade slightly and similarly a fatty acid dimer-based TPU while variability of degradation was noticed on a polycaprolactone-based TPU. On this last TPU, robust analysis of the degraded polymers showed that the *Penicillium* strain was the best degrading micro-organism. Membrane enzymes seemed to be involved in this degradation. It is the first time that a strain of *Penicillium* of the section Lanata-Divaricata displaying PU biodegradation ability is isolated. These newly discovered fungi are promising for the development of polyester PU waste management process.

2. Introduction

Material longevity associated with massive scale production makes synthetic polymers among the most abundant pollutants of our century. Studies attesting of their negative impacts on marine and terrestrial ecosystems are published at an increasing rate (Jambeck et al. 2015; Cao et al. 2017; Chae and An 2017). Polyurethanes (PU) as the 6th polymer with 18 Mt produced in 2016 (Furtwengler et al. 2017), are among the most pollutant polymers (Reddy et al. 2006). For instance, among the 70 plastic samples collected on a beach 39 were identified as PU (Turner and Lau 2016). PU are intended for long term applications: foams for construction and automotive industries, elastomers for sportswear or medical devices, coatings and sealants (Reulier and Avérous 2015). They are thus designed to resist environmental conditions. Management of PU

products end-of-life is currently inappropriate with most of PU wastes going to landfill and existing solutions such as incineration are not economically and environmentally satisfying. Thus, more high valued added and sustainable solutions for PU waste treatment are urgently needed.

Bioremediation is a general term to qualify the action of depollution using a biological entity while biological recycling implies obtaining valuable products from waste also using biological means. Polymer biodegradation is the basis for both processes (Wierckx et al. 2015). Briefly, micro-organisms secrete extracellular enzymes able to depolymerize the macromolecules leading to the formation of oligomers and monomers. Then, these molecules can be assimilated by micro-organisms and metabolized for biomass and energy production (Lucas et al. 2008). Degradation can also be performed by a single enzyme from microbial, vegetal or mammalian origins (Tokiwa, Suzuki, and Takeda 1988; Marchant et al. 1987; Brzeska et al. 2015). Bioremediation and biological recycling are attractive ways to fight against plastic pollution because of their low costs and environmental impacts (Azubuike, Chikere, and Okpokwasili 2016).

The biodegradation of PU materials is increasingly studied. PU are mainly synthesized by the reaction between polyols and polyisocyanates. The polyol is generally a polyester or a polyether. The micro-organization of PU with soft and rigid segments are one of the main key towards high performance materials (Carré et al. 2016). The building blocks involved in PU synthesis control the final macromolecular architecture, the crystallinity and the molar mass, which are parameters highly impacting the final PU biodegradability. For instance, polyester-based PU are known to be more biodegradable than polyether ones due to the hydrolysable ester bonds (Cregut et al. 2013).

Degradation of PU with filamentous fungi is described as more efficient than with bacteria due to outstanding enzymatic arsenals and abiotic features linked to filaments formation (Barratt et al. 2003; Lucas et al. 2008). Several types of PU materials such as polyether urethane foam (Alvarez-Barragan et al. 2016), thermoplastic polyester (Khan et al. 2017) and PU waterborne dispersion of polyester PU (Russell et al. 2011) have already been described as sensitive to fungal biodegradation.

Bottlenecks in PU biodegradation are the evaluation of the degradation with efficient techniques and the understanding of the degradation mechanisms. For instance, the majority of the micro-

organisms were tested on commercial model PU such as Impranil®, a waterborne polyester PU dispersion (Rowe and Howard 2002; Gautam, Bassi, and Yanful 2007). This polymer is a milky solution presenting the particularity of becoming clearer when hydrolyzed. Although Impranil® is largely studied because convenient for screening approaches, its chemical architecture is very specific and far to be representative of most of the common PU. Then, Impranil® is not a perfect model. When more complex PU substrates are used, a significant lack of robust methods of analysis is noticeable (Ibrahim 2009; Urgun-Demirtas, Singh, and Pagilla 2007). Degradation analysis must couple overall degradation evaluation with, for example, weight loss measurement and polymer surface analysis. A sufficient number of repetitions must also be considered.

In this publication, we achieved a screening of fungal strains isolated from PU-containing environments on several tailor-made TPU substrates, different models with different macromolecular architectures which are much more representative of the conventional PU, compared to Impranil®. Degradation of these model TPU represents a step towards the degradation of real and largely produced PU such as foams. Strains of fungi presenting the best degradation capacities were then identified. Analysis of the degraded polymers and enzymatic assays on the culture supernatants were then performed to suggest degradation mechanisms.

3. Materials and methods

3.1. Materials

3.1.1. Minimal medium preparation

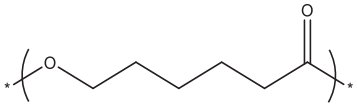
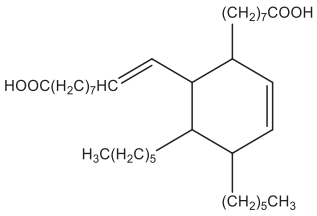
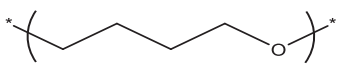
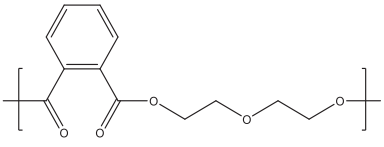
Minimal medium (MM) was prepared according to Alvarez-Barragan *et al.*, with some minor modifications (Alvarez-Barragan et al. 2016). Buffer with 19 mM of NaH_2PO_4 and 33.5 mM of K_2HPO_4 was supplemented by agar (15 g/L final) and HCl (1.8 mM final) and autoclaved at 121°C for 18 minutes. To 1 L of this buffer was added 10 mL of each of the following solutions, filtered at 0.22 µm with polystyrene filters (in this order): (i) a solution of $MgSO_4$ x $7H_2O$ (250 µM), (ii) a solution containing $FeCl_3x6H_2O$ (147 µM), $ZnCl_2$ (9 µM), $CoCl_2$ (6.5 µM), Na_2MoO_4 x $2H_2O$ (12 µM), $CaCl_2$x $2H_2O$ (10 µM), $CuCl_2$ x $2H_2O$ (13 µM), $MnCl_2$ x $2H_2O$ (14.6 µM), H_3BO_3 (12 µm) and finally,

(ii) a solution of NH_4SO_4 (7.6 mM). Streptomycin at 60 µg/mL final was added in the MM to prevent bacterial growth.

3.1.2. Polyurethane substrate

The waterborne polyester PU dispersion used for this experiment is Impranil-DLN®, kindly supplied by Covestro (Germany). Four representative TPU substrates with different macromolecular architectures (i.e., PCL-based TPU, fatty acid dimer-based TPU, polyether-based TPU, and poly(ester ether)-based TPU) was synthesized. The list of polyols used for TPU syntheses is presented in Table 3.2.1. The 4,4'-methylene diphenyl diisocyanate (4,4'MDI, Suprasec 1306 - Mn = 250.25 g/mol) was purchased from Huntsman (USA). The 1,4-butanediol (1,4-BDO, Mn = 90.12 g/mol) was purchased from Invista (USA).

Table 3.2.1 – Polyols used for TPU synthesis

Commercial name	Supplier of the polyols	Composition	Chemical structure of the main components of the polyols
Capa 2302	Perstorp	Polyester: polycaprolactone (PCL)	
Radia 7285	Oleon	Polyester based on fatty acid dimer from rapeseed oil and short diol	
Terathane 2000	Invista	Polyether: Polytetrahydrofuran (PTHF)	
Stepanpol PD56	Stepan	Polyester based on diethylene glycol and phthalic anhydride	

The TPU syntheses were performed into a jacketed reactor at 80°C under nitrogen gas flow. Stoichiometric conditions were used with a NCO/OH ratio of 1, using an equimolar quantity of the polyol and 1,4-BDO. A “one-step” method was performed. The 4,4’-MDI was warmed at 60°C and then introduced into the reactor followed by the mix of polyol and 1,4-BDO, previously heated at 80°C. Resulting TPU material was then compressed at 145°C with a plate press (Labtech Hot Press) to obtain thin films. They were then cut into pieces of 2 x 2 cm.

3.2. Methods

3.2.1. Fungal isolation

Six samples likely to contain PU-degrading fungi were collected: (i) a landfill stagnant water from a PU production plant collected in summer (Saint Julien du Sault, France), (ii) a floor mat collected in a junkyard (Oberhausbergen, France), (iii) a yellowish, old, PU foam collected in a PU production plant (Saint Julien du Sault, France), (iv) a two-years old standing water in contact with concrete slab covered by PU varnish (Strasbourg, France), (v) a PU foam collected in a discharge (Strasbourg, France) and (vi) a greenish PU foam from an old office chair (Strasbourg, France).

Solid samples were ground and sieved to obtain homogeneous fragments. Liquid samples were centrifuged and the pellets were recovered. Each sample was dispersed until adhesion on the four media used for fungal isolation: MM and Potatoes Dextrose Agar (PDA) with and without 2 g/L of Impranil-DLN®, kindly supplied by Covestro (Germany). PDA is a rich medium that allowed the recovery of a large number of fungi. On the contrary, MM provides extra sources of nitrogen and minerals to promote fungal growth while using the environmental sample or Impranil as carbon source. Growing fungi were successively plated until isolation. Strains isolated were finally plated on PDA for storage at 4°C.

3.2.2. Degradation assays

Degradation assays on the four synthesized TPU were performed in 40 mL sterile polystyrene flasks. Each flask was weighted beforehand. TPU pieces of 2 x 2 cm were washed twice with distilled water, once with ethanol 70% and dried overnight at room temperature under a laminar flow hood. One piece of TPU was then added into each flask and weighted. 2 mL of MM without

agar were added in each flask. Medium was inoculated using fungal agar plate culture and an inoculation loop of 1 µL.

After 2 months of incubation at 30°C, cultures were stopped. Supernatants were recovered for biochemical assays, if necessary. TPU pieces were washed twice with distilled water, once with ethanol 70%, dried overnight at room temperature and finally, weighted. Negative control consisted in TPU pieces incubated in liquid MM without fungal inoculation. For the initial screening, cultures were performed with only one repetition while the confirmation step was performed with at least four repetitions for each pair of fungus/TPU.

3.2.3. Fungal identification

Selected strains were grown on PDA for 3 days at 30°C and sent to the accredited service for fungi identification of CABI (UK). All procedures were validated and the processing was undertaken in accordance with CABI's in-house methods for filamentous fungi and yeasts. Strains were identified by Internal Transcribed Spacer (ITS) rDNA sequence analysis and in one case, partial beta-tubulin gene sequence analysis. Several algorithms were used for identification: the BLAST algorithm with the CABI Validated Sequence Database for Fungi, the FASTA algorithm with the Fungus database from EBI and the BLAST algorithm with the 'Others' database from NCBI, limited to type strains. Strains were deposited in CABI collection.

Nikon A1R laser confocal microscope (Nikon, Japan) equipped with a 100x Plan Fluor water immersion objective was used for observation of filaments and spores of fungi.

3.2.4. Characterization techniques

Scanning electronic microscopy (SEM) was used to study the evolution of the surface morphology of the degraded films. Vega-3 (Tescan) apparatus in high vacuum mode with working distances in the range of 6-8 mm and an acceleration voltage of 5 kV was used. Before examination, samples were coated with a thin layer of gold with a gold coater (Quorum Q 150 RS, Quorum Technologies).

Infrared spectroscopy was performed with a Fourier transformed infrared spectrometer Nicolet 380 from Thermo Scientific (US) used in reflection mode equipped with an ATR diamond module

(FTIR-ATR). An atmospheric background was collected before each sample analysis (32 scans, resolution 4 cm^{-1}). FT-IR analyses were performed on the washed samples after the two months of incubation.

Size exclusion chromatography (SEC) was used to determine the evolution of the polymers molar masses. TPU was dissolved in tetrahydrofuran (THF) at room temperature and filtrated at 0.2 µm with polypropylene membranes directly in vials. An Acquity-APC (Waters) in THF at 40 °C was used for SEC analysis. Three columns (Acquity APC XT 450 Å 2.5 µm 4.6x150 mm, 200 and 45) were connected. 10 µL of dissolved polymer solution were injected. A run of 11 minutes with a flow of 0.6 mL/min was applied. A calibration curve using polystyrene (PS) standards was built for molar mass determination.

3.2.5. Enzymatic assays

The supernatants of the degradation assays were recovered and centrifuged 10 min at 10,000 g in order to remove residues of fungi. pNitrophenyl acetate (pNPA, Sigma-Aldrich) and pNitrophenyl hexanoate (pNPH, TCI Europe) were prepared at 55 mM in dimethylsulfoxyde (DMSO). In a microcuvette, 10 µL of substrate in DMSO were added to 690 µL of MM and 300 µL of centrifuged supernatant. Absorbance was measured at 415 nm at room temperature. The molar absorptivity of pNitrophenol under these assay conditions was 57.4 $mM^{-1}.cm^{-1}$. Activities were expressed as µmol of pNitrophenol.$s^{-1}.mL^{-1}$ of supernatant.

4. Results

4.1. Screening and identification of the fungi

Strains isolated were named according to the type of agar plate used for isolation (I = Impranil + PDA, P = PDA, MMI = Impranil + MM and MMP = MM) followed by a digit indicating the origin of the sample (see materials and methods). Twenty-seven strains were isolated. Three were yeast shaped while the others were filamentous fungi.

The collection of fungi was then screened on four TPU. No significant weight loss was detected for the ethylene glycol and phthalic anhydride based-TPU which is a poly(ester ether)-based TPU and for the PTHF-based TPU (polyether-based TPU) (Figure 3.2.1a). Evident weight losses were measured for the PCL-based TPU and the fatty acid dimer-based TPU incubated with 16 strains from the collection (weight loss > 1% for at least one of the two polymers). The best strains were selected for further analysis. The strains P2a1 and MMP3b (Figure 3.2.1) were selected because of weight loss higher than 3% on the PCL-based TPU after two months of incubation. MMP3c1 was chosen because of the similar weight loss measured on the PCL-based TPU and on the fatty acid dimer-based TPU. The degradation efficiency determined with 5 replicates was confirmed for these three strains. They displayed weight losses coherent with those from the screening (Figure 3.2.1b). Weight losses of 3.2, 8.9 and 1.5% were measured for P2a1, MMP3b and MMP3c1 respectively for the PCL-based TPU. Weight losses of 1.7, 1.3 and 1.8% were measured for the fatty acid dimer-based TPU. Fungal growth was observed on the two polymers with the three strains (Figure S3.2.7 & Figure S3.2.8). P2a1 which was isolated from a floor mat collected in a junkyard and MMP3b and MMP3c1, isolated from a yellowish PU foam collected in a PU production plant, were thus selected as strains of interest for PU biodegradation.

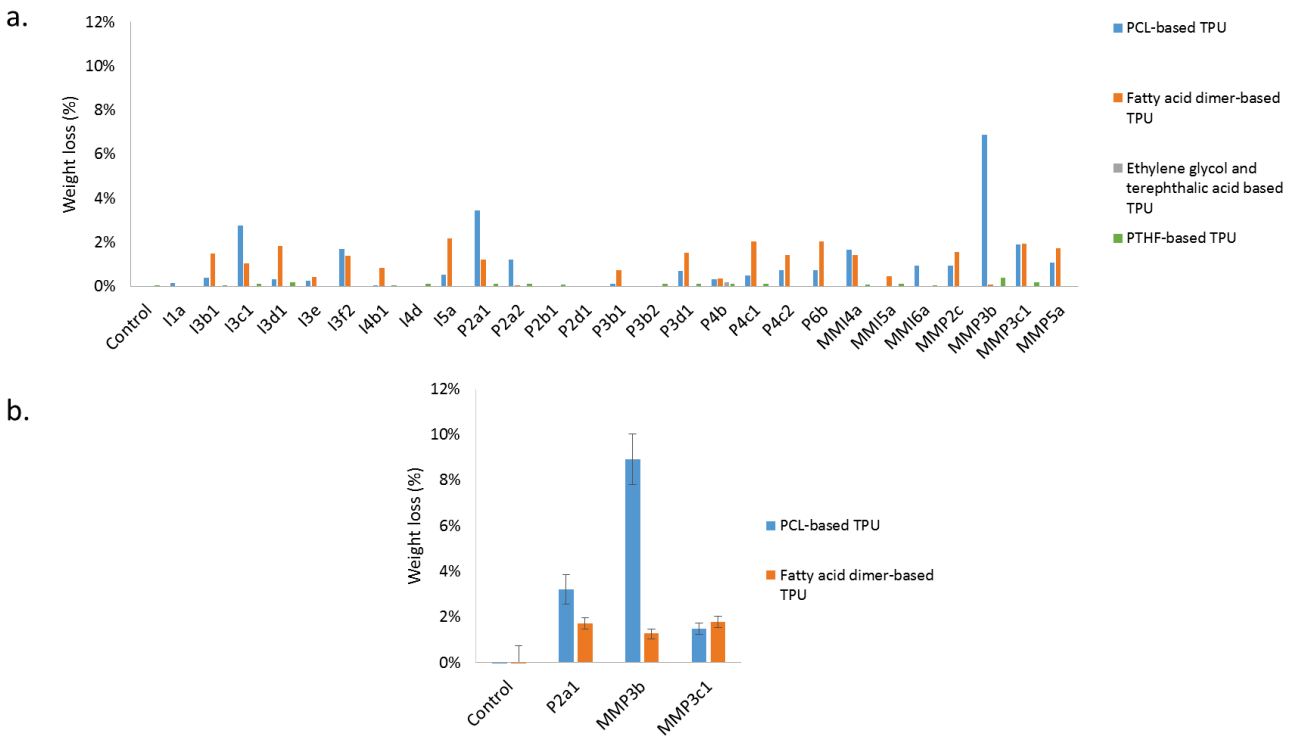


Figure 3.2.1 – Evaluation of the weight losses on the different TPU (a) screening of the collection of fungi and (b) control of the activity of the 3 selected strains

The selected fungi were then identified using molecular and morphological tools. Molecular identification allows to the definition of the genus and also an orientation toward a section within the genus. The strain P2a1 was identified as member of the genus *Alternaria* (CABI deposit number: 506837). Using FASTA from EBI, the ITS sequence (NCBI accession: MH410558) matched at 98-99% identity to members of species of the genus *Alternaria* (Hou et al. 2016). Using the BLAST algorithm with the 'Others' database from NCBI, limited to sequences from type strains, this sample showed matches at 99% identity to type strains from species of *Alternaria* . There was no clear distinction between species from the sequence results, hence identification was given at genus level. Morphological observation tends to support such identification since P2a1 conidia morphologies and growth aspects on PDA shown in Figure 3.2.2 are similar to those of the strains of *Alternaria sp.*, , described in Matsumyia *et al.*, (Matsumiya et al. 2010). The strain MMP3b ITS (NCBI accession: MH410559) sequence matches at 97.7-99.6% identity to sequences from

members of *Penicillium* section Lanata-Divaricata. This result was confirmed by partial beta-tubulin sequencing (NCBI accession: MH594856) which also gave top matches to members of this group including 98.2% identity to sequence GU981629 from the type strain of *P. brasilianum*, which have been published in the ICPA List of *Penicillium* names and in Visagie *et al.* (Visagie et al. 2014). Morphological similarities of the conidia can be observed between Figure 3.2.2 and the *P. brasilianum* observation from Inokoshi *et al*., and Cho et al (Inokoshi et al. 2013; Cho, Hong, and Go 2005). This strain was deposited in CABI collection (CABI deposit number: 506838). The ITS sequence of MMP3c1 showed 100% identity to sequence AF027863 from the type strain of *A. flavus* and to sequence EF661560 from the type strain of *A. oryzae*, both of which have been published in the ICPA List of *Aspergillus* names and in Peterson *et al.* (Peterson 2017). No distinction can be made between these two species. Identification has thus been made to the section at genus level (CABI deposit number: 506839).

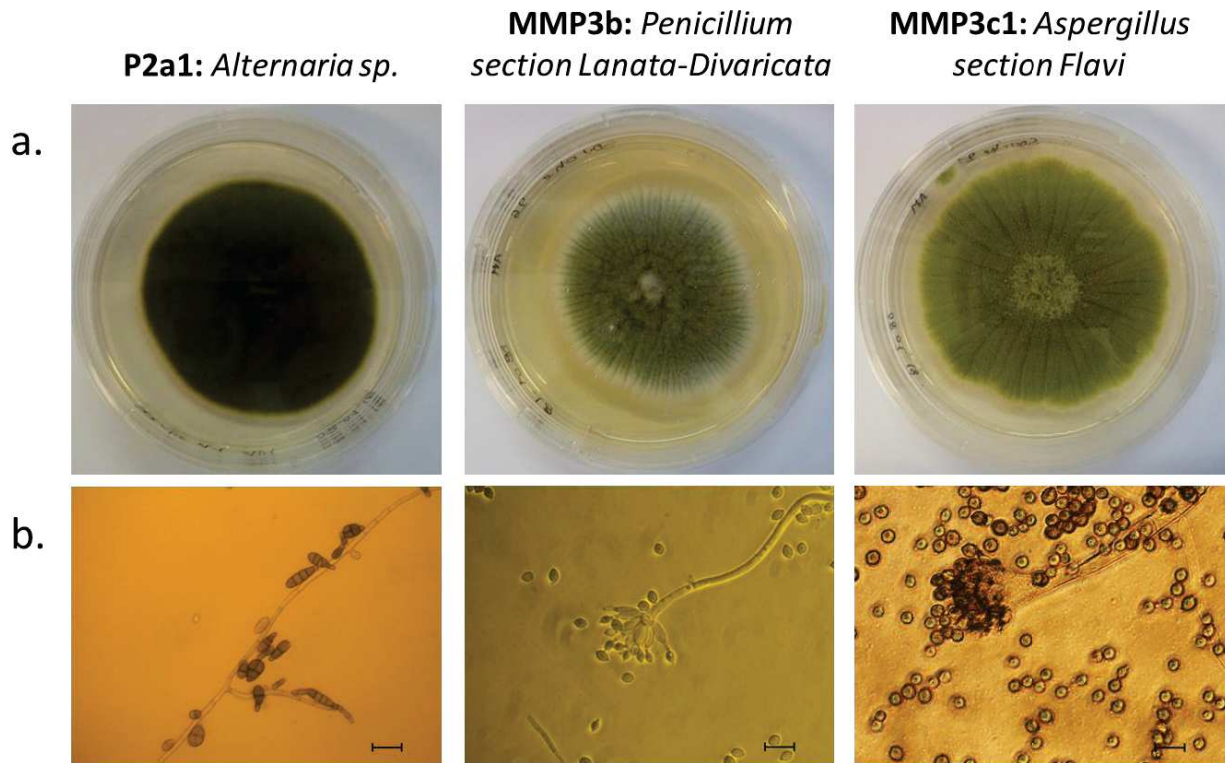


Figure 3.2.2 – Strains selected for TPU biodegradation (a) observation on PDA after 4 days of growth at 30°C and (b) optical microscope observations of hyphae and conidiophores/conidia (Bars represent 25 µm)

4.2. Materials analysis

Several techniques were used to deepen the characterization of the degraded TPU. One replicate of each experiment was examined by SEM (Figure 3.2.3 & Figure 3.2.4). PCL-based TPU incubated without fungi displayed a smooth and intact surface (Figure 3.2.3). Incubation with the strain of *Alternaria* showed deep holes disseminated in few places of the polymer surface. Incubation with the strain of *Penicillium* revealed a regular network of excavations. Finally, the strain of *Aspergillus* led to an irregular surface with shallow cracks.

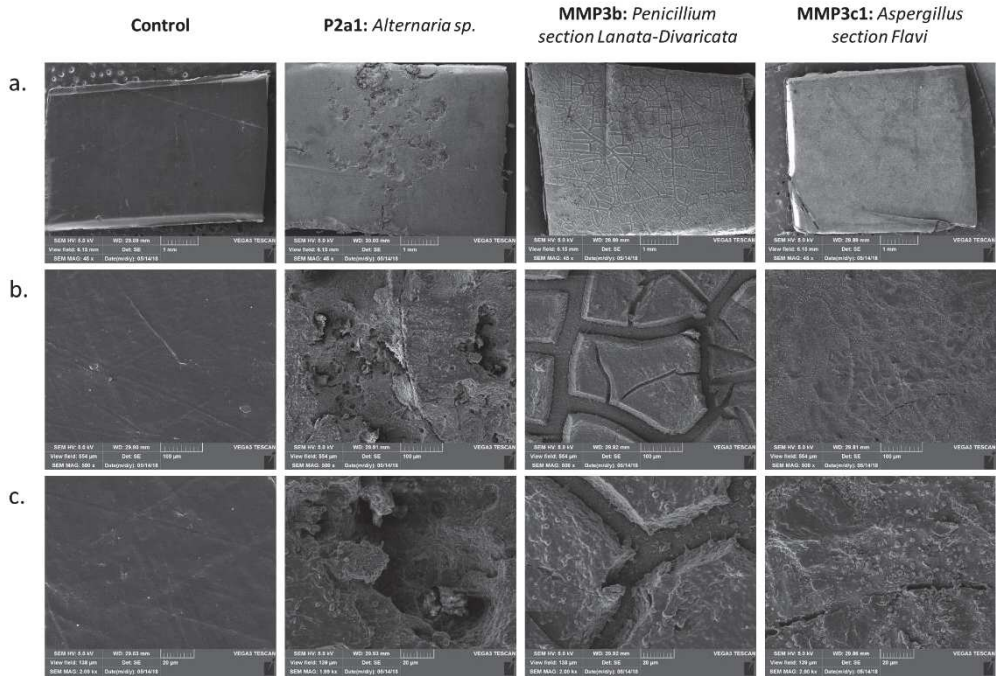


Figure 3.2.3 – Observation of PCL-based TPU pieces incubated 2 months at 30 °C (a) magnification of 45 (bars represent 1 mm), (b) magnification of 500 (bars represent 100 µm) and (c) magnification of 2000 (bars represent 20 µm)

Fatty acid dimer-based TPU incubated without fungi showed also an undamaged surface (Figure 3.2.4). Whitish zones were observed when incubated with the three strains. Higher magnification on these zones revealed superimposed patches for the three degraded samples. Cracks were shallow. Only the superficial layer of the polymers was impacted.

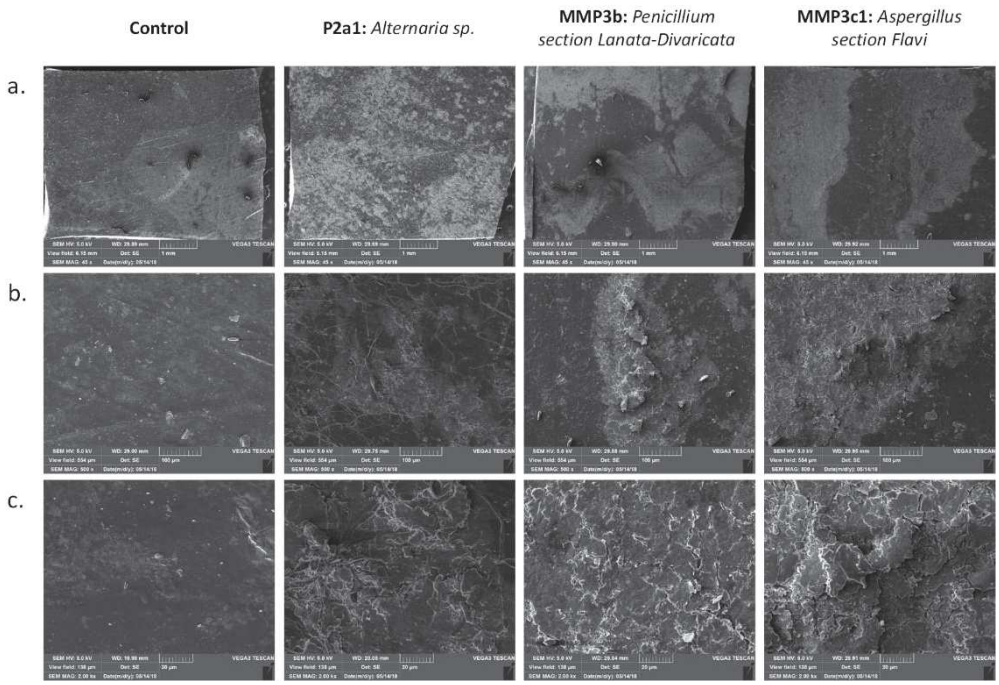


Figure 3.2.4 – Observation of fatty acid dimer-based TPU pieces incubated 2 months at 30 °C (a) magnification of 45, (b) magnification of 500 and (c) magnification of 2000

FT-IR was performed on all the samples to characterize the chemical modifications on TPU surfaces. Spectra, available on supplementary data (Figure S3.2.9 & Figure S3.2.10), allowed the identification of three zones of interest corresponding to ester or urethane bonds. A wide band between 3 325 and 3 340 cm^{-1} corresponds to OH and NH stretching without differentiation between these two moieties (Spontón et al. 2013; Oprea 2010) (Figure S3.2.11). Peaks at 1720 cm^{-1} are attributed to C=O of an ester bond while signal at 1700 cm^{-1} can be attributed to C=O of a carboxylic acid (Schmidt et al. 2017; Elzein et al. 2004; Osman et al. 2018). The band at 1530 cm^{-1} is generally attributed to urethane/amide N-H bending vibration in the secondary amide (Oceguera-Cervantes et al. 2007; Spontón et al. 2013; Oprea 2010). PCL used for the PCL-based TPU synthesis was also separately analyzed (Figure S3.2.12). No signal appeared on this zone for this polyester. In order to perform a semi-quantitative study, the intensity of peaks of interest were normalized by the intensity of the stable peak at 2 865 cm^{-1} corresponding to $–CH_2$ stretching (Figure 3.2.5). For the PCL-based TPU, an increase of the signal intensities at 1530 and 3330 cm^{-1} and a decrease of the signal intensity at 1720 cm^{-1} were noticed for TPU incubated with

fungi, compared to the control. A band at 1700 cm^{-1} appeared for TPU incubated with *Penicillium*. For the fatty acid dimer-based TPU, signals intensities increase at 1530 and 3330 cm^{-1} and decrease at 1720 cm^{-1} were also observed but to a lesser extent. The peak at 1700 cm^{-1} appeared for all the TPU incubated with fungi.

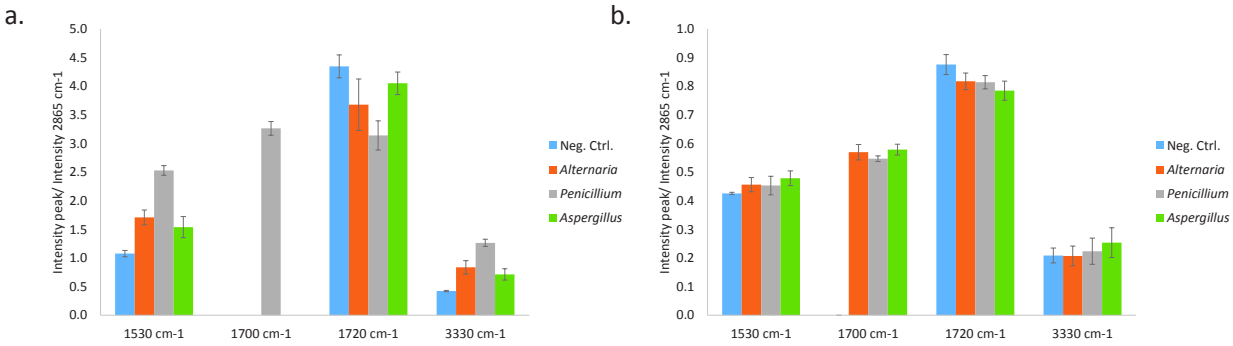


Figure 3.2.5 – Semi-quantitative analysis of FT-IR (a) for the PCL-based TPU and (b) for the fatty acid dimer-based TPU

All the degraded polymers were then analyzed by SEC to evaluate the evolution of the molar masses distributions. Chromatograms are available on Figure S3.2.13 & Figure S3.2.14. Two zones can be distinguished: the main polymer chain consisting in the main peak (between 4 and 7 minutes of retention time) and the oligomers corresponding to smaller peaks (between 7 and 8.5 minutes of retention). Number average molar mass (Mn), mass average molar mass (Mw), dispersity (Đ) and the percentage area of the main peak were collected in Table 3.2.2. For the PCL-based TPU, variability between the replicates increased after fungal degradation (up to 116,000 ± 13,000 g/mol) while high repeatability was observed for the negative control (125,000 ± 1,000 g/mol). No significant variation of Mn, Mw or Đ was noticed between PCL-based TPU incubated with and without fungi (p-value > 0.05). Only a slight decrease of dispersity was observed for the strain of *Penicillium* (p-value = 0.044). A decrease of the relative area of the main polymer chains was noticed for the three strains. For the fatty-acid based TPU, a significant increase of the Mn from 35,000 for the control to 37,000, 40,000 and 38,000 was observed for the strains of *Alternaria*, *Penicillium* and *Aspergillus*, respectively (p-value < 0.05). A significant decrease of the Mw was noticed from 120,000 g/mol for the negative control to 104,000, 108,000

and 101,000 g/mol, for the strains of *Alternaria*, *Penicillium* and *Aspergillus,* respectively (p-value < 0.05). The variation of Mn and Mw involved a high decrease of the corresponding dispersities. As for the PCL-TPU, the relative area of the main peak also decreased significantly.

Table 3.2.2 – SEC analysis of degraded polymers (Mn and Mw expressed as g/mol)

		PCL-based TPU				**Fatty acid dimer-based TPU**			
		Neg. Ctrl.	*Alternaria*	*Penicillium*	*Aspergillus*	Neg. Ctrl.	*Alternaria*	*Penicillium*	*Aspergillus*
Mn	Mean	58,000	55,000	55,000	57,000	35,000	37,000	40,000	38,000
	Std dev	0	2,000	6,000	3,000	1,000	1,000	1,000	1,000
	p-value		0.116	0.419	0.366		0.003	0.000	0.010
Mw	Mean	125,000	122,000	116,000	121,000	120,000	104,000	108,000	101,000
	Std dev	1,000	8,000	13,000	11,000	4,000	3,000	6,000	4,000
	p-value		0.439	0.148	0.267		0.001	0.017	0.001
Disp	Mean	2.17	2.21	2.10	2.13	3.43	2.79	2.70	2.69
	Std dev	0.01	0.05	0.04	0.08	0.06	0.05	0.10	0.14
	p-value		0.258	0.044	0.193		0.000	0.000	0.000
Area	Mean	99.5%	99.0%	98.5%	99.2%	99.0%	96.3%	94.9%	94.8%
	Std dev	0.0%	0.1%	0.5%	0.1%	0.1%	0.3%	0.3%	1.0%
	p-value		0.000	0.002	0.002		0.000	0.000	0.000

4.3. Enzymatic assays

Esterase activity was determined on two substrates, pNitrophenyl acetate (pNPA) and pNitrophenyl hexanoate (pNPH), on the supernatant media of fungal cultures on PCL-based TPU and fatty acid dimer-based TPU (Figure 3.2.6). Both chemicals release pNitrophenol after hydrolysis of the ester bonds. Measured activities were significantly higher for the fatty acid dimer-based TPU than for the PCL-based TPU. Enzymes of *Alternaria* and *Penicillium* produced during the degradation of the fatty acid dimer based-TPU showed a better affinity for the

substrate with 6 aliphatic carbons. Esterase activity of the supernatants of culture on PCL-based TPU was the highest for *Alternaria* and null for *Penicillium*.

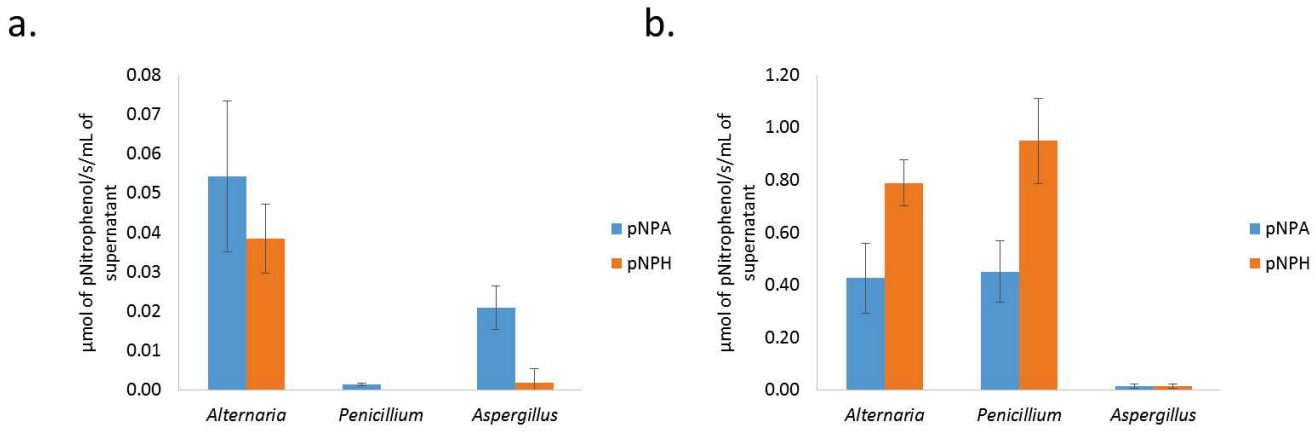


Figure 3.2.6 – Evaluation of esterase activity with pNPA and pNPH on the supernatant of fungal cultures of (a) PCL-based TPU and (b) fatty acid dimer-based TPU

5. Discussion

To be as close as possible to conventional PU wastes, the synthesized polymers were based on 4,4'MDI, thus providing highly resistant urethanes groups (Sabbioni et al. 2012). Screening on TPU only showed degradation of the two polyester PU after two months of incubation at 30°C with the polymers as the sole carbon source (Figure 3.2.1). The poly(ester ether) PU and the polyether PU were already described as not degradable using efficient PU-degrading enzymes (Magnin et al., unpublished results). For the poly(ester ether) PU, it seems that the macromolecular structure makes the ester bonds not easily accessible for enzymes. The degradation of the polyester PU was also confirmed by the evaluation of the fungal growth (Figure S3.2.7 & Figure S3.2.8). Degradation ability of the fungi thus depends on the bonds of the soft segment of the TPU but also on the macromolecular structure. Usually, additional carbon sources such as glucose (Khan et al. 2017; Matsumiya et al. 2010) or PDB (Alvarez-Barragan et al. 2016), are added to promote the microbial growth and thus, the biodegradation. Regarding the development of an efficient

remediation or bio-recycling process, working without additional carbon source such as glucose, as in our case, is a major financial gain.

Identifications of the strains were done at the genus level for the strain P2a1 which is *Alternaria sp.* and to the section for MMP3b and MMP3c1 which are respectively *Penicillium* section Lanata-Divaricata and *Aspergillus* section flavi. *Alternaria* species have already been described for PU degradation. For example, a strain of *A. tenuissima* was reported as active on pyridine-based polyether PU (Oprea et al. 2018). *A. solani,* isolated from soil, showed degradation activity toward a polyester PU (Ibrahim 2009). *Alternaria sp.* was described for the degradation of a polyether PU foam and on model molecules with a urethane bond as a sole cleavable bond (Matsumiya et al. 2010). *Aspergillus flavus* has been described as capable of degrading a polyester PU as sole carbon source (Mathur and Prasad 2012) while *A. oryzae* has never been described on PU.

The strain involving the most significant weight loss on TPU is the strain of *Penicillium* section Lanata-Divaricata. Isolated species of *Penicillium* have been described on PU degradation studies such as *Penicillium chrysogenum* (Alvarez-Barragan et al. 2016), but to the best of our knowledge, no isolated strains of *Penicillium* belonging to the section Lanata-Divaricata has been reported to degrade PU. It is thus the first time for such a strain that a PU degrading activity is clearly demonstrated. The best match for our strain appeared to be with *P. brasilianum.* This species is widely known as an enzyme factory, mainly for production of cellulases (Jorgensen et al. 2004; Jørgensen and Olsson 2006) and feruloyl esterases (Panagiotou, Olavarria, and Olsson 2007).

Complete analysis of the degradation systems was provided for the two polyester PU incubated with the three strains of interest. Different degradation profiles were observed and followed by weight loss, SEM, FT-IR analysis and enzymatic assays for the PCL-based TPU incubated with the three strains (Figure 3.2.1b, Figure 3.2.3 & Figure 3.2.4). SEC data did not provide differential results (Table 3.2.2).

Surface analysis of the PCL-based TPU incubated with *Penicillium* by SEM revealed a specific network like a fungal mycelium's one. Moreover, Figure 3.2.2b shows the filaments and spores of the strain of *Penicillium*, with filaments diameters from 5 to 10 µm. The network displayed rift widths slightly higher than 10 µm, strengthening the hypothesis of material excavation

corresponding to filament growth on the polymer surface. The absence of extracellular esterase activity indicates that enzymes mainly responsible of the TPU degradation seem to be cell-bound proteins. These types of enzymes have already been described on the bacterial degradation of PU (Akutsu et al. 1998).

Better affinity for the pNPH, ester substrate with 6 aliphatic carbons, was expected for the enzymes produced within the culture medium with the PCL-based TPU as it consists in the repetition of an ester bound to a chain of six carbons. Surprisingly, none of the three strains produced enzymes with better affinity for this substrate. However, for the fatty acid dimer-based TPU, the activity on pNPH was higher than on pNPA. This could be linked to the hydrophobic chain length of the pNPH ester group matching the one of the pendant alkyl chains of the polyester segment of this TPU.

The weight loss for the fatty acid dimer-based TPU was low (about 1.5%) and almost similar for all three strains (Figure 3.2.1b). Surface analysis performed by SEM and FT-IR displayed similar polymer surface morphologies and similar changes in surface moieties (Figure 3.2.4 & Figure 3.2.5). Enzymatic assays showed close esterase activities between the strain of *Alternaria* and *Penicillium* while a significantly lower esterase activity for the strain of *Aspergillus* (Figure 3.2.6b). The mechanisms implemented by the strain of *Aspergillus* seemed to be different than the ones of *Alternaria* and *Penicillium*. Despite these differences, the three strains presented analogous impact on the fatty acid dimer-based TPU.

In addition to strain comparison, this study allows also discussing the techniques used to evaluate the polymer degradation. For the fatty acid dimer-based TPU, SEC analysis of the remaining solid polymer showed decreasing Mw and increasing Mn highlighting some polymers alterations. This increase in Mn values could be explained by the limited cleavage of the polymer chains along with the release of very short oligomers. These small molecules could be lost by solubilization in the surrounding medium or, in the case of fungal degradation, by their metabolization by the micro-organisms. It has been reported that even a very limited loss in the lower molar mass fractions may result in a significant apparent increase in Mn values (Mohd-Adnan, Nishida, and Shirai 2008). The loss of material is limited. This is highlighted by the decrease of main polymers and

the evolution of the oligomers concentration (Table 3.2.2). These oligomers could correspond to the observed superimposed patches observed by SEM at the polymer surface (Figure 3.2.4). As oligomers remained mainly associated to the polymer pieces, the weight loss was limited even when the observed degradation was significant. The same behavior was observed on fatty acid dimer-based TPU after 50 days of degradation at 37°c with an esterase (Magnin et al., unpublished). For the PCL-based TPU, no significant change in molar mass distributions was observed on the remaining solid polymer and only low amount of oligomers were found, most probably due to their consumption by fungi. SEC appears thus to be a good technique for the analysis of polymers showing superficial "damages", as a complement of the weight loss measurement, but seems less relevant for highly degraded polymers.

This work offers a clear proof of the interest of FT-IR for the semi-quantitative evaluation of TPU biodegradation. For the PCL-based TPU, *Penicillium* showed the highest peak intensity increase at 3300 cm^{-1} and 1700 cm^{-1} and the highest decrease at 1720 cm^{-1} while displaying the highest weight loss whereas *Aspergillus* showed the lowest intensity variations at these three wavelengths. For the fatty acid dimer-based TPU, comparable but lower weight losses were observed with all three strains. However, FT-IR spectra showed a slight increase in peak intensity at 3300 cm^{-1} and a decrease at 1720 cm^{-1}. The intensity variation for these two peaks is thus consistent with the weight loss and thus with overall polymer degradation. However, the interpretation of the increase of the peak intensity at 1530 cm^{-1} appears trickier. Oprea *et al.* suggested that an increase of this signal is related to urethane bond hydrolysis (Oprea 2010) while others suggested that a decrease of this signal attests for urethane bond degradation (Oceguera-Cervantes et al. 2007; Sarkar and Lopina 2007). The mechanism and location of the hydrolysis is most likely the reason for the contradictory statements found in the literature. An esterase activity would lead to the hydrolysis of the ester bond part of the urethane group, resulting in more carbamate groups exposed and thus increased signal of secondary amide band. In the case of protease activity the amide bond part of the urethane group would be cleaved resulting in the decrease of the corresponding band signal. The observed increase of the secondary amide signal tends to evidence that the esterase activity is predominant. But, either an increase or a decrease of this signal would attest for changes in the polymer structure. Consequently, fungal

degradations have a clear impact on the urethane bond but no definitive conclusion can be made on the exact nature of this impact. This point needs additional investigations.

Identification of these three strains, and particularly the strain of *Penicillium*, is the premise for the development of bioremediation and biological recycling processes. Understanding the full metabolisms involved in the biodegradation should then be fully elucidated with, for example, proteomic tools. Optimization of the conditions must be carried out to improve TPU degradation. For instance, pre-incubation in rich media could be tested to further improve the biodegradation. More generally systems optimization might allow to degrade other polyurethanes, such as crosslinked ones e.g., PU foam, which represent the large majority of the commercial PU. This paper lays the foundation for an effective and green solution toward relieving the burden of polyurethane waste pollution, which is in the core of one of the major and very actual concern linked to the worldwide plastic pollution.

Acknowledgments

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7. Supporting information

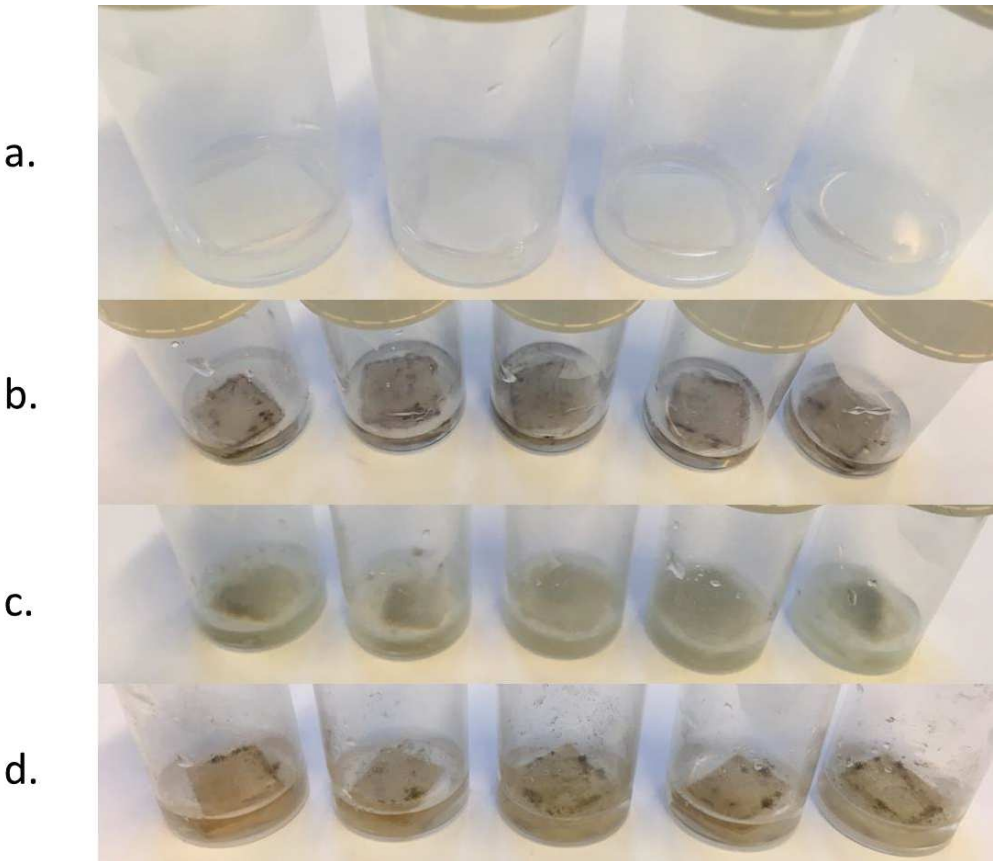


Figure S3.2.7 – Evaluation of the fungal growth on the PCL-based TPU after 2 months of incubation at 30°C (a) without fungi, (b) with the strain of Alternaria, (c) with the strain of Penicillium and (d) with the strain of Aspergillus

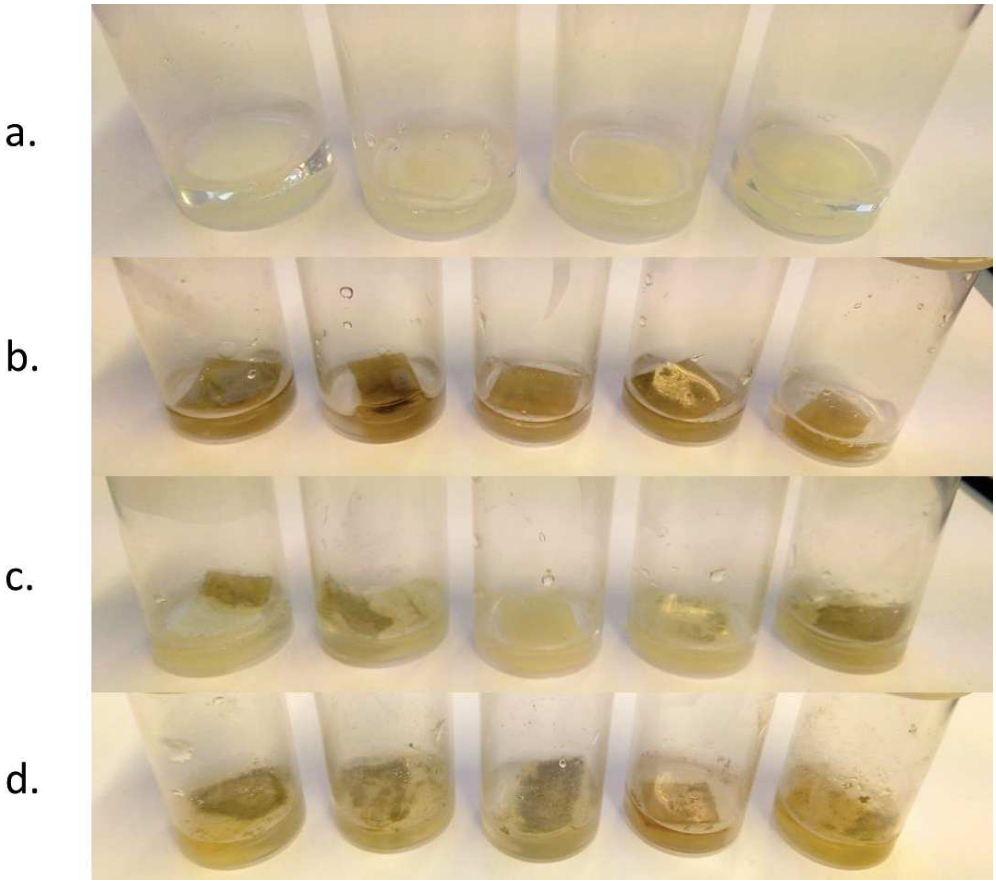


Figure S3.2.8 – Evaluation of the fungal growth on the fatty acid dimer-based TPU after 2 months of incubation at 30°C (a) without fungi, (b) with the strain of Alternaria, (c) with the strain of Penicillium and (d) with the strain of Aspergillus

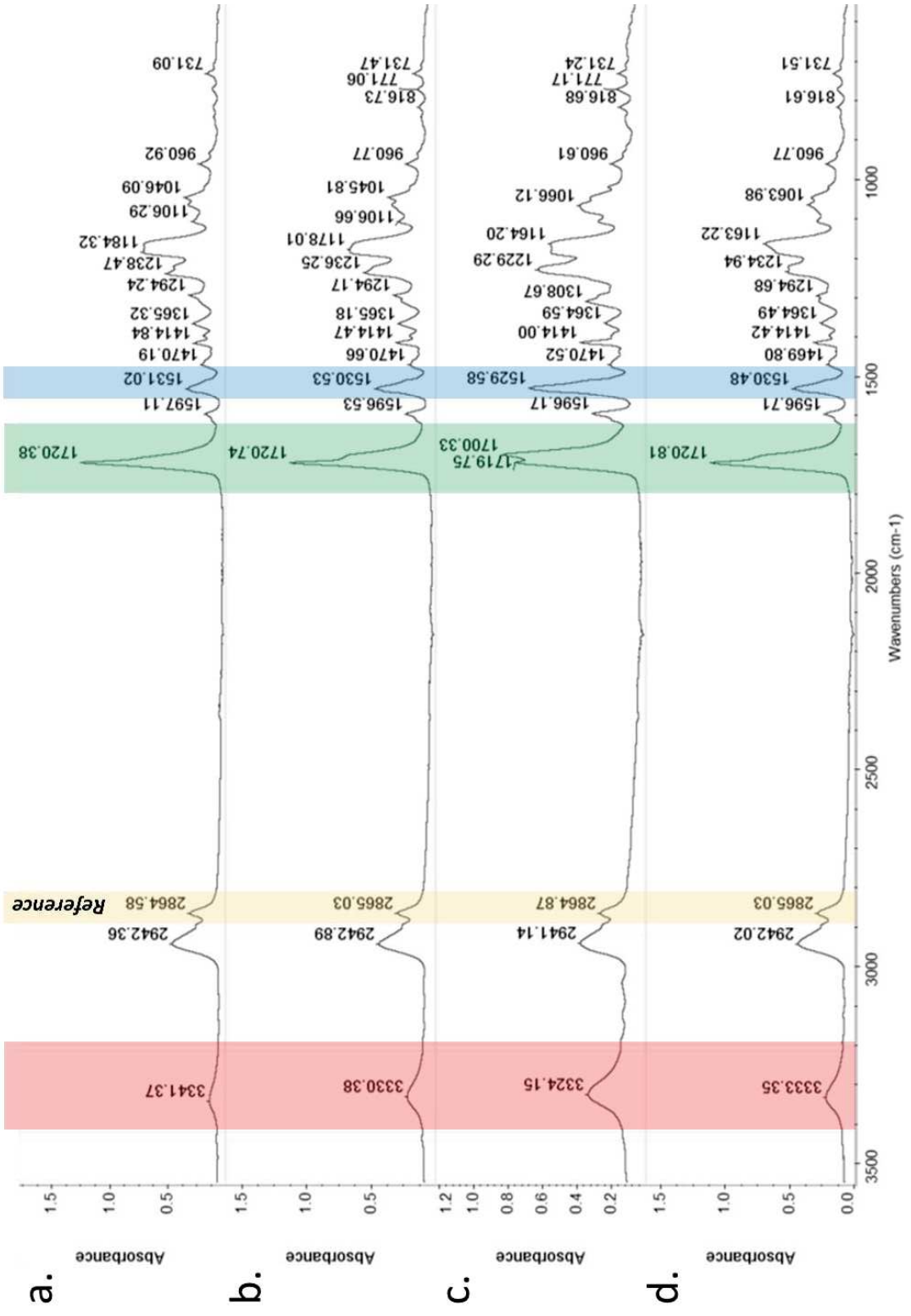


Figure S3.2.9 – Absorbance spectra of FT-IR analysis for PCL-based TPU incubated 2 months at 30°C a) without fungi, (b) with the strain of Alternaria, (c) with the strain of Penicillium and (d) with the strain of Aspergillus

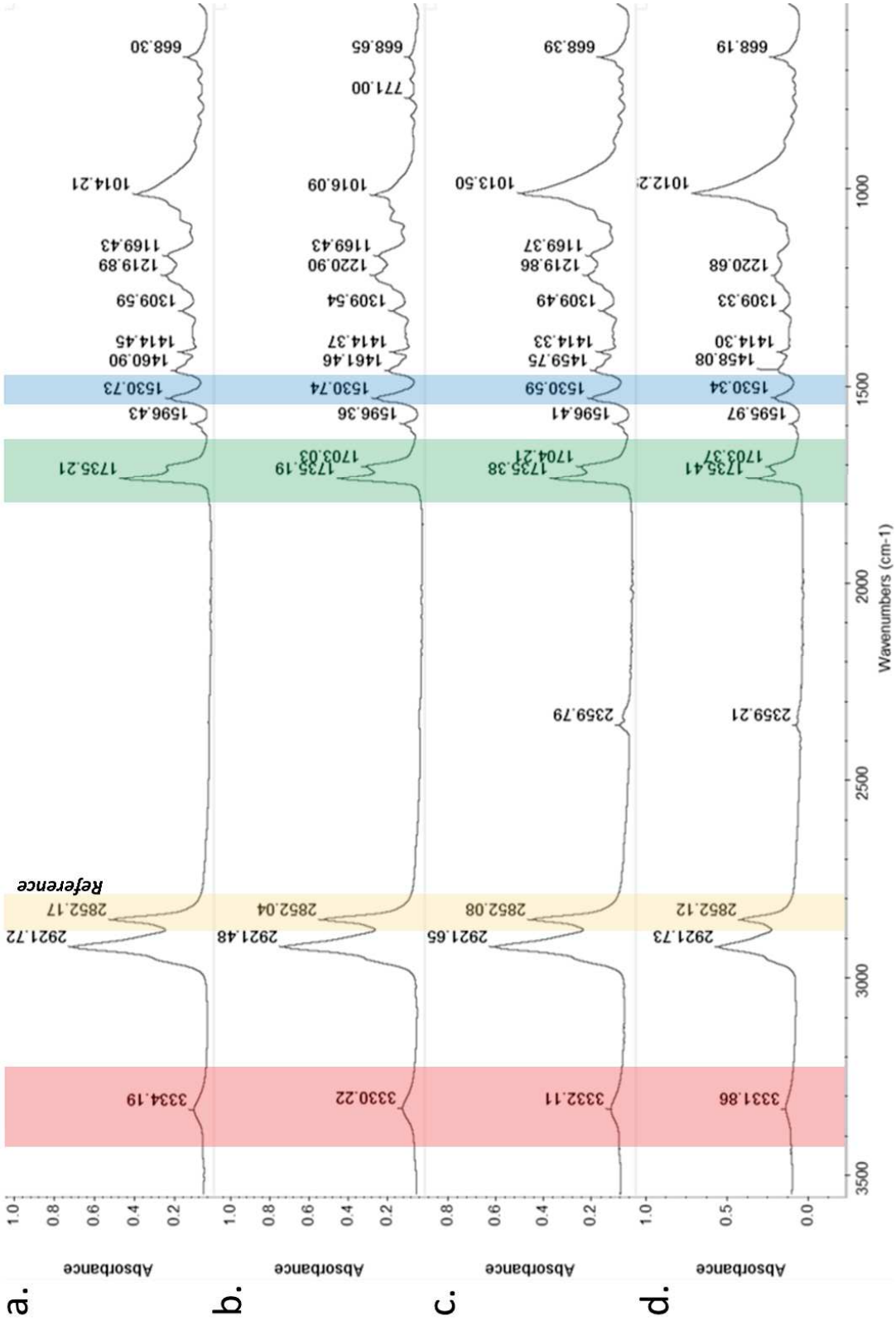


Figure S3.2.10 – Absorbance spectra of FT-IR analysis for fatty acid dimer-based TPU incubated 2 months at 30°C a) without fungi, (b) with the strain of Alternaria, (c) with the strain of Penicillium and (d) with the strain of Aspergillus

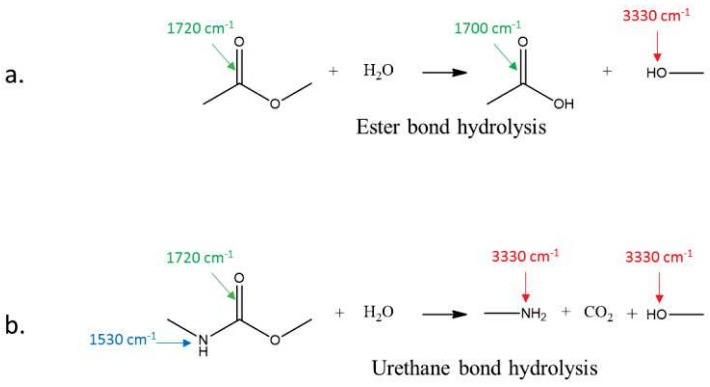


Figure S3.2.11 – FT-IR signals attribution to (a) ester and (b) urethane bonds hydrolysis

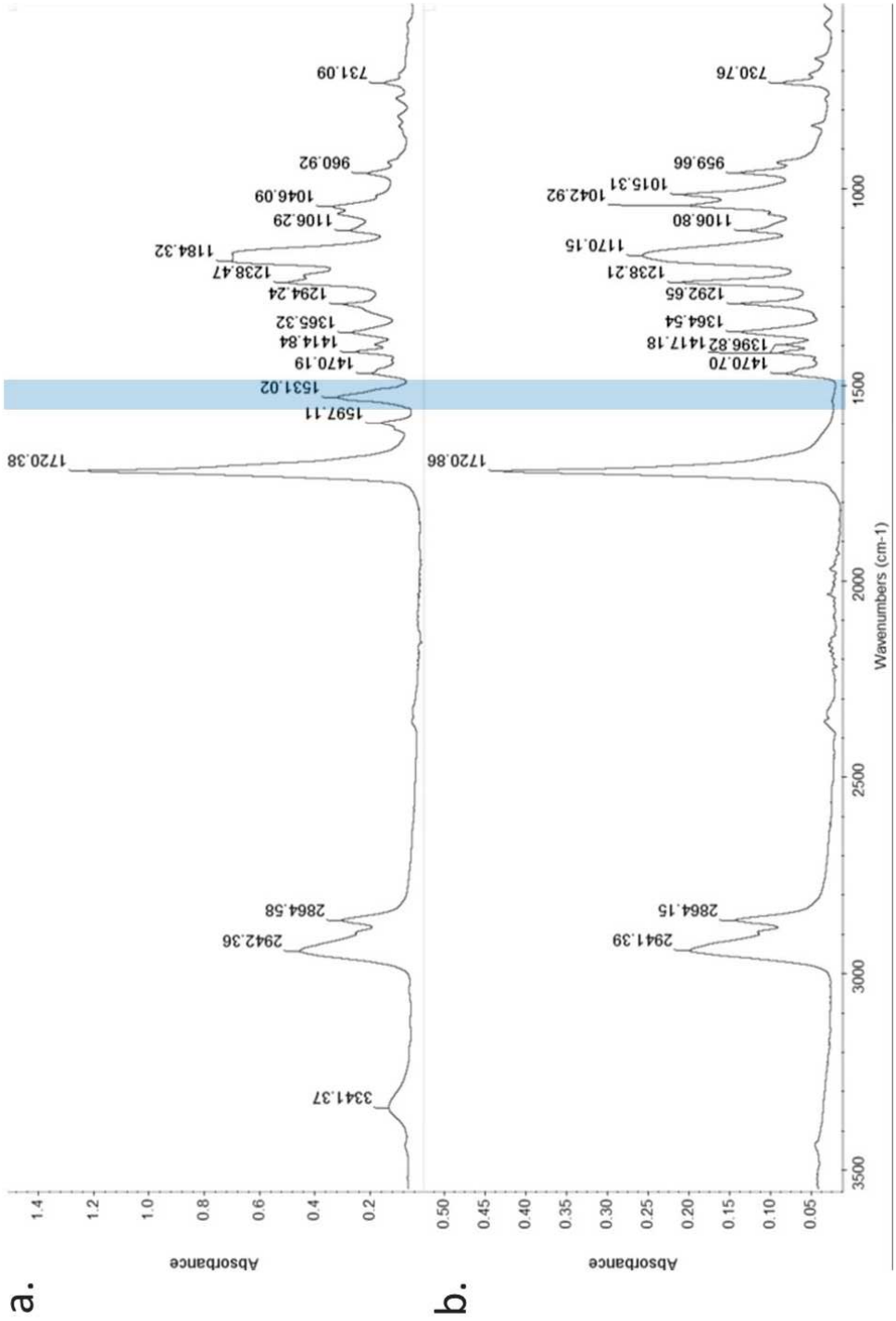


Figure S3.2.12 – Comparison of absorbance FT-IR spectra of (a) the PCL-based TPU and (b) the PCL polyester used for the synthesis of the PCL-based TPU

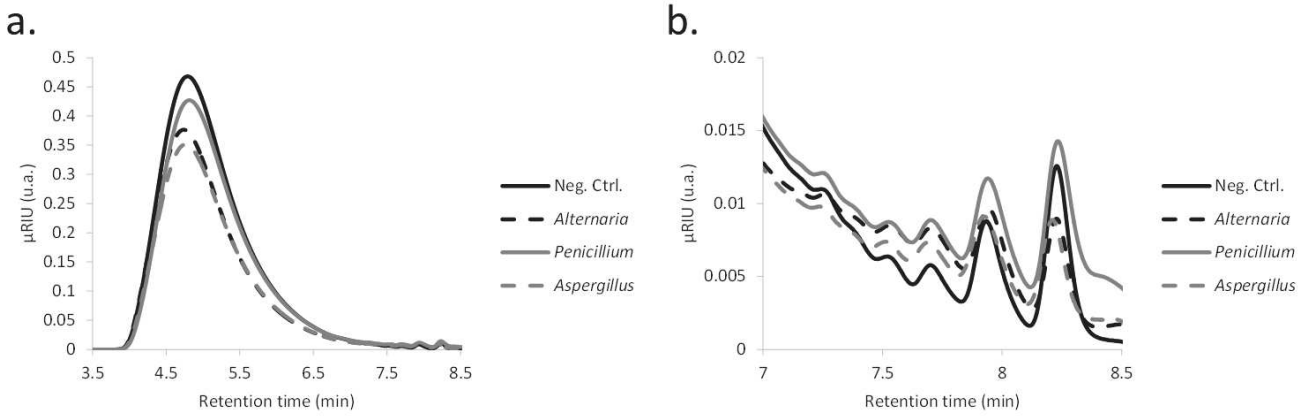


Figure S3.2.13 – SEC analysis of the PCL-based TPU incubated 2 months at 30°C with fungi (a) full chromatogram and (b) focus on oligomers

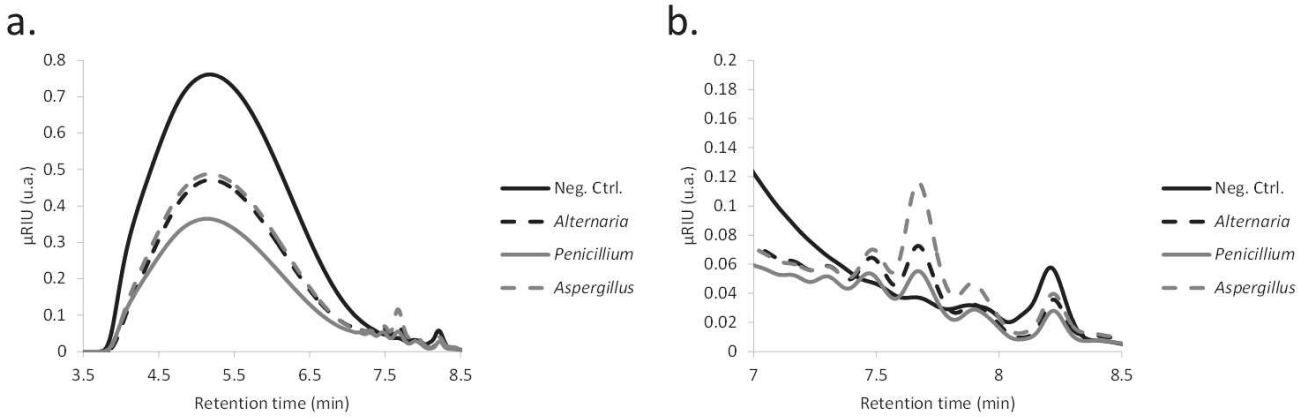


Figure S3.2.14 – SEC analysis of the fatty acid dimer-based TPU incubated 2 months at 30°C with fungi (a) full chromatogram and (b) focus on oligomers

Sous-chapitre 3.3. Expériences complémentaires : Analyse des produits de dégradation

Afin d'élucider et comprendre les mécanismes impliqués dans la dégradation des TPU par les champignons, nous avons tenté d'identifier les produits de dégradation présents dans les surnageants de culture. Comme décrit dans le sous-chapitre 2.2, nous avons utilisé une méthode de chromatographie liquide couplée à la spectrométrie de masse (MS). Cette stratégie a eu pour but de détecter le 4,4'-MDA et ses dérivés potentiels. En effet, la présence de 4,4'-MDA permet de confirmer le clivage des liaisons uréthanes du TPU utilisé.

L'analyse du 4,4'-MDA seul révèle un signal observable entre 6,5 et 7 minutes d'élution sur le chromatogramme MS, accompagné d'un pic UV à environ 220 nm lié aux cycles aromatiques (Figure 3.3.1). En MS, la fragmentation permet de résoudre la structure chimique des molécules analysées. Les fragments se forment en fonction de la facilité avec laquelle les liaisons peuvent se rompre au sein d'une molécule. Par exemple, une liaison carbone-oxygène, nécessite une énergie de collision plus faible pour se rompre qu'une liaison carbone-carbone. La fragmentation à 20 eV du 4,4'-MDA donne un ion unique de 106,07 Da. Cette molécule semble correspondre à la rupture du 4,4'MDA au niveau du méthylène entre les deux cycles aromatiques.

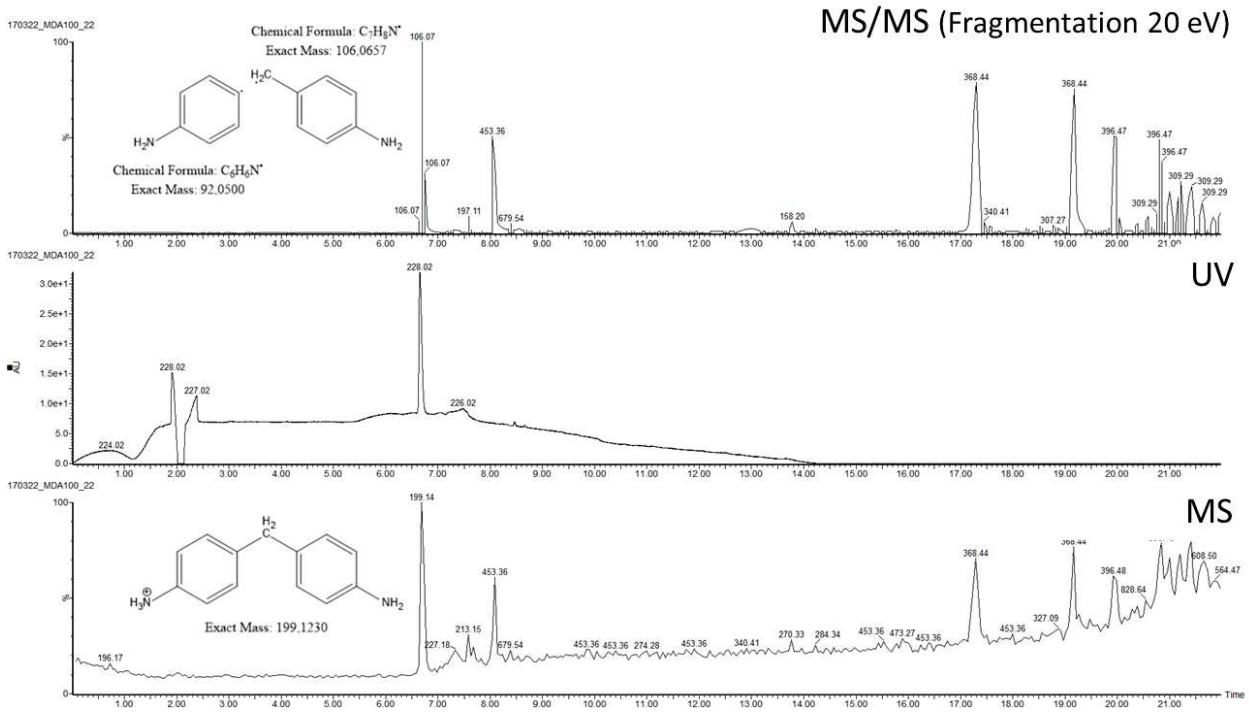


Figure 3.3.1 – Chromatogrammes MS, UV et MS/MS (fragmentation à 20 eV) du 4,4'-MDA

Trois cultures ont été réalisées avec les souches d'*Alternaria, Penicillium et Aspergillus* décrites dans le sous-chapitre précédent. Ces champignons ont été incubés 2 mois à 30°C en présence de PCL TPU dans les conditions décrites précédemment. Les surnageants ont ensuite été prélevés et analysés. La présence de 4,4'-MDA n'a été relevée dans aucun des échantillons étudiés. Cependant, une série d'ions de masses différentes et donnant un pic en UV aux alentours de 220 nm a été observée pour les 3 échantillons (Figure 3.3.2). L'échantillon issu de l'incubation avec la souche de *Penicillium* présente le plus d'ions.

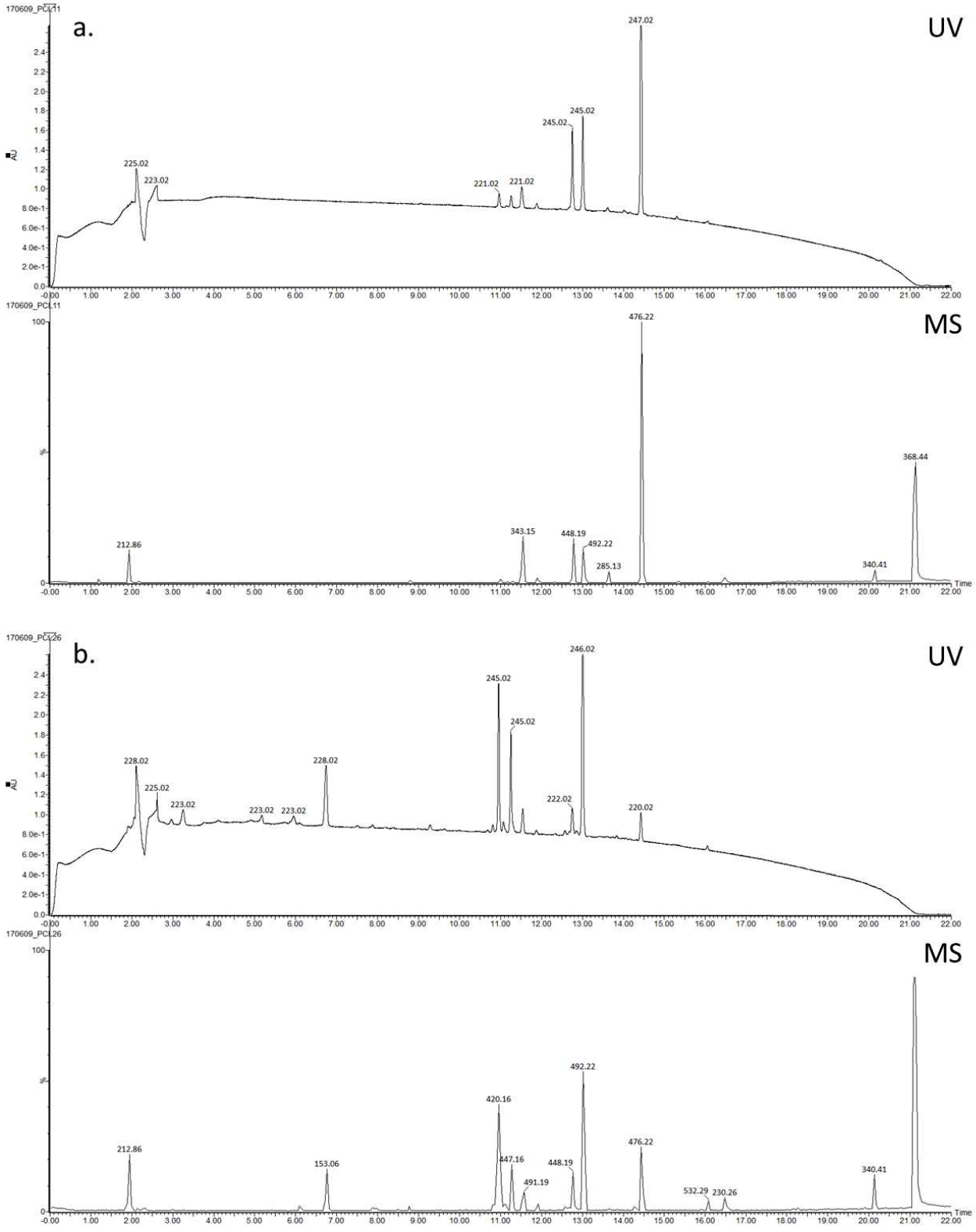


Figure 3.3.2 – Chromatogrammes MS et UV pour la souche (a) d'Alternaria, (b) de Penicillium et (c) d'Aspergillus

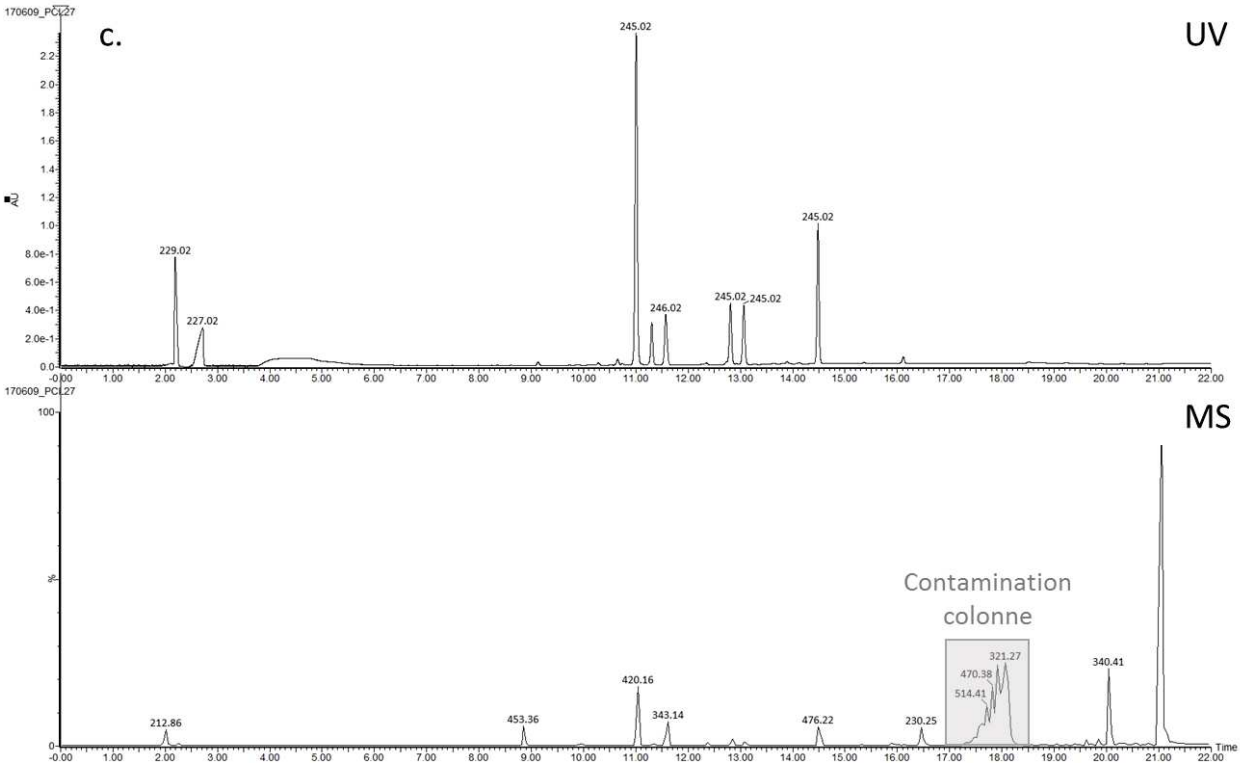


Figure 3.3.2 – Chromatogrammes MS et UV pour la souche (a) d'Alternaria, (b) de Penicillium et (c) d'Aspergillus (suite)

Les ions présents sur le chromatogramme MS et corrélés à un pic UV ont été relevés. Pour évaluer leur intensité, les aires des pics des chromatogrammes UV ont été utilisées (Figure 3.3.3). Les pics à 420,14, 447,14, 476,20 et 492,20 Da sont les plus intenses et sont présents dans les trois surnageants. Ces ions peuvent correspondre à des molécules issues de la dégradation du TPU base PCL par les champignons mais également à des produits des métabolismes fongiques secrétés dans ces conditions de culture.

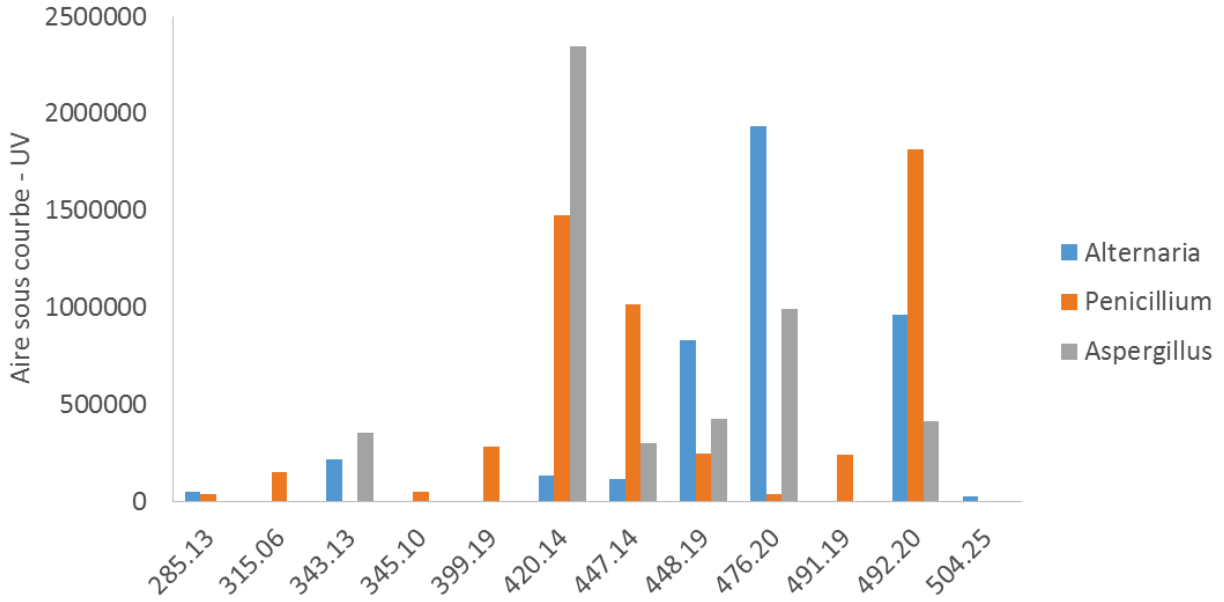


Figure 3.3.3 – Intensité des pics UV des ions de différentes masses détectés dans les surnageants de culture

Afin d'élucider la structure de ces ions, leurs spectres de fragmentation ont été étudiés (Figure 3.3.4). La présence d'un pic à 106.06 Da identique à celui du spectre de fragmentation du 4,4'-MDA est observé dans les spectres de fragmentation des ions de masses 472,20 et 496,20 Da (en rouge sur la Figure 3.3.4). Ces ions semblent donc être des dérivés de 4,4'-MDA. La présence d'un fragment de 150,06 Da, commun aux quatre ions (en jaune) et d'un fragment de 132,05 Da commun à trois ions (en bleu) montre qu'un motif commun existe entre ces ions. Il semblerait donc que les ions majoritairement présents dans les surnageants soient des produits de dégradation, potentiellement dérivé du 4,4'-MDA, et non des produits des métabolismes fongiques. Malgré de nombreuses tentatives, aucune structure totalement satisfaisante et inspirée de la structure initiale du polymère n'a pu être proposée.

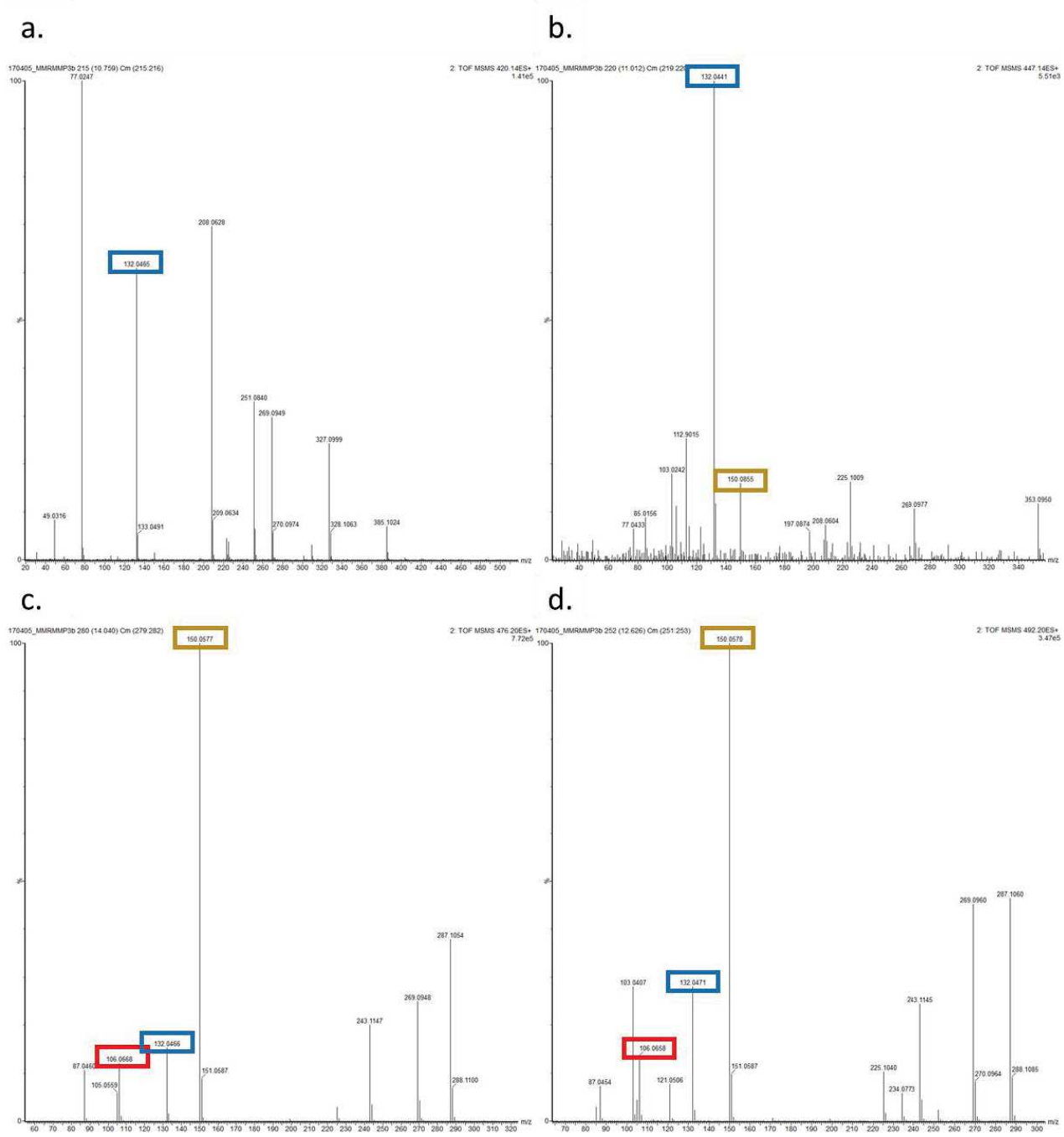


Figure 3.3.4 – Spectres de fragmentation des ions de masses (a) 420,14, (b) 447,14, (c) 476,20 et (d) 492,20 Da

L'analyse des surnageants de culture fongique sur TPU avait pour objectif d'aider à la compréhension des mécanismes de dégradation. Nous nous sommes focalisés sur la présence de 4,4'-MDA et de ses dérivés afin d'étudier le clivage de la liaison uréthane. Deux hypothèses

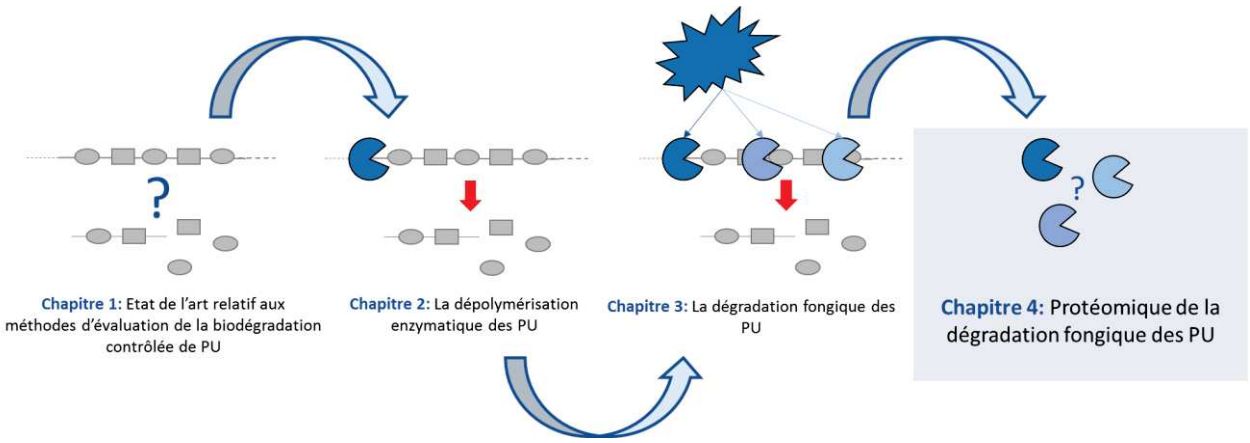
permettent d'expliquer l'absence de 4,4'-MDA dans les surnageants. Il est bien évidement possible que les champignons n'aient pas la capacité de rompre les liaisons uréthanes du TPU et ne soient donc pas capables de générer du 4,4'-MDA. Il est également envisageable que du 4,4'-MDA soit généré par clivage des liaisons uréthanes dans les cultures de champignons mais qu'il soit ensuite métabolisé par ces derniers, le rendant indétectable dans les surnageants. La présence de molécules dérivées du 4,4'-MDA montre que le segment rigide semble effectivement être affecté par la dégradation fongique. Cependant, la résolution des structures exactes des ions n'ayant pu être totalement menée, la rupture des liaisons uréthanes ne peut être confirmée avec certitude par cette expérience.

Conclusion

L'objectif de ce chapitre était d'identifier et de caractériser des souches de champignons présentant des compétences de dégradation sur les PU. Une collection de 27 souches a été établie à partir d'échantillons prélevés dans des environnements exposés longuement à des PU. Un criblage basé sur des cultures fongiques de 2 mois a été développé directement sur des substrats TPU complexes. A l'issu du criblage, trois souches ont montré des compétences élevées de dégradation sur deux polyesters TPU, l'un à base de PCL et l'autre à base de dimère d'acide gras. Ces souches ont été identifiées au genre pour la souche d'*Alternaria* et à la section du genre pour les souches de *Penicillium* section Lanata-Divaricata et *Aspergillus* section Flavi. Les trois souches ont une activité similaire sur le TPU à base de dimère d'acide gras. Des analyses de surface sur les polymères dégradés réalisées par microscopie et FT-IR ainsi que des analyses par SEC ont confirmé que la souche de *Penicillium* est la plus efficace sur le TPU base PCL. L'activité de dégradation de ce champignon sur ce polymère semble être liée à des enzymes pariétales alors qu'*Alternaria* et *Aspergillus* sécrètent des estérases permettant la dégradation.

Des analyses complémentaires ont été réalisées sur les surnageants de culture des trois champignons en présence du TPU base PCL. Des produits de dégradation dérivés du 4,4'-MDA ont été identifiés par spectrométrie de masse suggérant une dégradation du segment rigide du polymère. Cependant, l'absence de 4,4'-MDA dans les surnageants et l'absence de structures des produits de dégradation ne permet pas de confirmer le clivage des liaisons uréthanes.

Chapitre 4. Protéomique de la dégradation fongique des polyuréthanes



Introduction

Le chapitre précédent a permis de mettre en évidence trois souches de champignons dégradant des poly(ester uréthane). Dans une perspective de fin de vie et de recyclage des PU, la dégradation enzymatique s'avère plus pertinente que la dégradation microbienne car les produits de la dégradation peuvent être valorisés. Afin d'identifier de nouvelles enzymes efficaces, nous avons souhaité caractériser de manière plus approfondie les enzymes produites par les champignons lors de la dégradation des TPU. Cette démarche a aussi pour but de mieux comprendre les mécanismes de dégradation fongique.

Il est possible d'analyser l'ensemble des protéines sécrétées dans une culture microbienne grâce à une analyse de l'exoprotéome par spectrométrie de masse. Pour réaliser ces expériences, une collaboration a été initiée avec l'Institut pluridisciplinaire Hubert Curien (IPHC) et plus particulièrement avec le Laboratoire de Spectrométrie de Masse BioOrganique (LSMBO).

Une étude de faisabilité a d'abord été réalisée et fait l'objet du premier sous-chapitre. La démarche retenue, ainsi que les résultats obtenus sont ensuite présentés sous forme d'une publication constituant le reste du chapitre.

Sous-chapitre 4.1. Expériences préliminaires

La dégradation de polymères par des microorganismes est le résultat de l'action d'enzymes extracellulaires qui permettent des coupures de chaines ou dépolymérisation. De nombreux microorganismes dégradant les polyuréthanes (PU) ont été identifiés. Des tests enzymatiques sont fréquemment réalisés sur le surnageant des cultures microbiennes pour caractériser les activités enzymatiques de dégradation mais les enzymes elles-mêmes ne sont que rarement isolées et identifiées (Khan et al. 2017). Pour isoler l'enzyme présentant l'activité d'intérêt, un test sur substrat modèles tels que le pNitrophénol butyrate (pNPB) pour l'enzyme TfCut (Chen et al. 2008), l'Impranil pour PulA (Ruiz and Howard 1999; Vega, Main, and Howard 1999) ou encore le poly(diéthylène glycol adipate) pour PudA (Nomura et al. 1998) ont été développés. Une fois l'activité enzymatique détectée, l'enzyme peut ensuite être séquencée. Cette démarche est fastidieuse car l'enzyme doit être présente en quantité suffisante et correctement purifiée. De plus, l'utilisation d'un test sur substrat modèle limite les possibilités d'identification d'enzymes potentiellement efficaces sur des substrats plus complexes.

L'analyse de l'exoprotéome par spectrométrie de masse est une technique permettant d'identifier l'intégralité des enzymes extracellulaires dans une culture. Cette méthode est fréquemment utilisée pour étudier les enzymes surexprimées par des microorganismes pour la dégradation de la paroi végétale (Fernandez-Fueyo et al. 2016; Phalip et al. 2005). En effet, les gènes codant ces enzymes sont très majoritairement surexprimés en présence des biopolymères à dégrader. A notre connaissance, un seul article mettant en jeu cette technique pour l'étude de la dégradation de polymères synthétiques a été publié à ce jour (Wallace et al. 2017). Cette publication traite d'un substrat polyester biodégradable le poly(1,4-butylène adipate-co-téréphthalate) (Touchaleaume et al. 2018). Notre approche propose, pour la première fois, de considérer un polymère synthétique non-biodégradable. Il convient donc d'évaluer en premier lieu la faisabilité d'une telle étude.

Globalement, nos échantillons se prêtent particulièrement à ce type d'analyse car le polymère peut être utilisé comme seule source de carbone. En effet, un milieu minimum est utilisé et donc aucun autre signal n'induit, a priori, la production de protéines ayant une activité de

dépolimerisation. Les milieux de culture classiques contiennent des protéines interférant avec la mesure en spectrométrie de masse ce qui n'est pas le cas de notre milieu minimum.

L'étude de faisabilité a été réalisée sur un nombre restreint d'échantillons. L'objectif était de vérifier la comptabilité entre nos conditions de culture et l'analyse de l'exoprotéome. Nous avons sélectionné les souches d'*Aspergillus* et de *Penicillium*. Ces deux souches ont été incubées avec les deux polyesters TPU, décrits dans les chapitres précédents, synthétisés à partir d'un polyester à base de dimère d'acides gras (DFA) ou de polycaprolactone (PCL).

Après 3 semaines d'incubation à 30°C, les surnageants de culture ont été récupérés et filtrés avec des filtres de 0.2 µm pour éliminer les résidus de champignons et ne collecter que les protéines extracellulaires (Figure 4.1.1). Ces protéines sont ensuite digérées par la trypsine, une enzyme hydrolysant les protéines après les acides aminés basiques (lysine ou arginine). Ces peptides trypsiques générés sont analysés en spectrométrie de masse. La confrontation des masses mesurées et des masses théoriques, résultant de la digestion tryspsique *in silico* de bases de données protéiques, permet d'identifier les protéines présentes dans l'échantillon de départ. Il est ensuite possible de quantifier les protéines présentes dans les surnageants via des logiciels dédiés tel que Maxquant ou Proline.

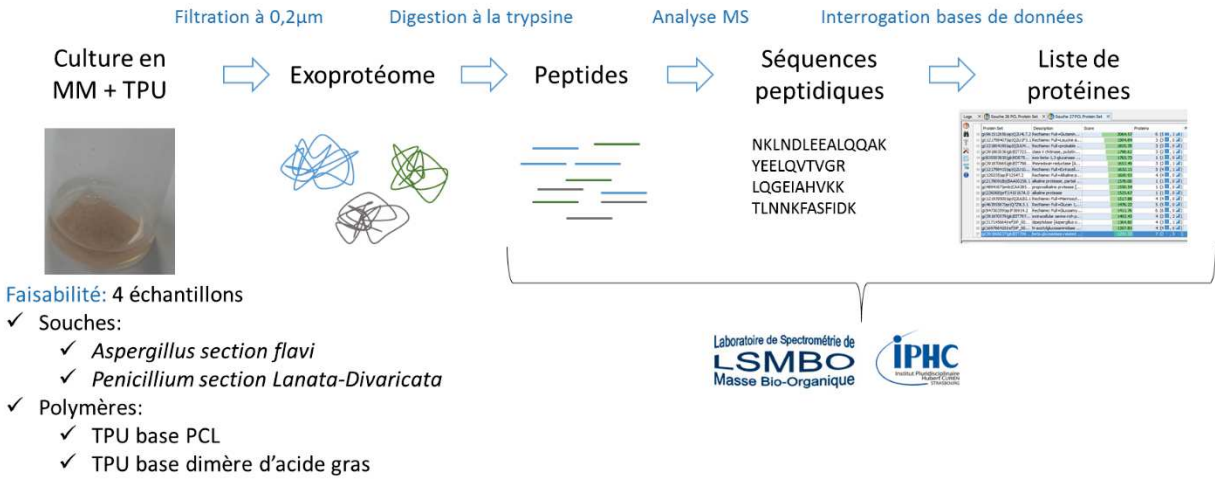


Figure 4.1.1 – Déroulé de l'étude afin d'd'identifier les enzymes mises en jeu.

L'interrogation des bases de données a fourni une liste de 219 protéines pour *Aspergillus* en présence de PCL TPU, 205 protéines pour *Aspergillus* en présence de DFA TPU, 205 protéines pour *Penicillium* en présence de PCL TPU et enfin, 114 protéines pour *Penicillium* en présence de DFA TPU. L'obtention d'un grand nombre de protéines dans chaque culture confirme la possibilité d'utiliser une analyse d'exoprotéome sur nos souches de champignons cultivées sur des TPU.

La majorité des protéines identifiées pour *Aspergillus* correspondent à l'espèce *Aspergillus oryzae*. La majorité des protéines identifiées pour *Penicillium* correspond à l'espèce *Penicillium brasilianum*. Ces résultats sont cohérents avec l'identification moléculaire réalisée sur ces souches et présentée dans le chapitre 3, sous chapitre 2. Cette étude nous permet donc d'affiner l'identification des souches fongiques isolées lors de ce travail.

Il convient désormais d'affiner les objectifs pour la suite de ces travaux. En effet, une analyse différentielle entre plusieurs conditions d'essai doit être mise en place pour faire ressortir les enzymes d'intérêt. Il serait pertinent d'ajouter, en sus des TPU base polyesters, les polyesters composant les TPU. La différence entre polyesters TPU et polyesters étant la partie uréthane, une analyse différentielle pourrait mettre en exergue des enzymes spécifiques de la dégradation de cette partie. De plus, le temps d'incubation pour cette phase a été déterminé arbitrairement. Il conviendra de l'optimiser.

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Sous-chapitre 4.2. Exoproteome analysis of *Aspergillus oryzae* and *Penicillium brasilianum* grown on neat polyesters and polyurethane substrates

Audrey Magnin[1], François Delalande[2], Aurélie Hirschler[2], Eric Pollet[1], Vincent Phalip[3], Christine Carapito[2], Luc Avérous[*,1]

(1) BioTeam/ICPEES-ECPM, UMR CNRS 7515, Université de Strasbourg, 25 rue Becquerel, 67087 Strasbourg Cedex 2, France

(2) Laboratoire de Spectrométrie de Masse Bio-Organique, IPHC, UMR 7178, CNRS-Université de Strasbourg, ECPM, 67087 Strasbourg, France

(3) Univ. Lille, INRA, ISA, Univ. Artois, Univ. Littoral Côte d'Opale, EA 7394 - ICV - Institut Charles Viollette, 59000 Lille, France

(*) Corresponding author: luc.averous@unistra.fr

1. Abstract

An efficient approach to identify polyurethane (PU)-degrading enzymes from filamentous fungi is presented. Exoproteomes of *Aspergillus oryzae* and *Penicillium brasilianum*, PU-degrading fungi, grown on two neat polyesters and two polyester-based thermoplastic PU (TPU) based on polyester polyols were studied. Mechanisms involved in the degradation of the two TPU by *P. brasilianum* are too different to perform a differential analysis of the exoproteome. Among the 184 proteins identified for *A. oryzae*, 22 enzymes, mainly hydrolases and oxydoreductases, appeared to be specific of the growth on TPU when comparing to the growth on polyesters, glucose and cellulose. Four peptidases seem to be good candidates for the urethane bond cleavage.

2. Introduction

Polyurethanes (PU) are a family of versatile synthetic polymers. These polymers are intended for a wide range of materials such as foams, elastomers or coating. With 18 Mt produced in 2016, PU rank as the 6th most produced polymers in the world (Furtwengler et al. 2017). To make PU sustainable materials, from cradle to the grave, both the synthesis of biobased polymers and the development of efficient PU waste management processes to control their end of life, are major challenges to address in a close future. This problematic is unfortunately clearly highlighted with the actual strong concerns of oceans pollution where PU-based micro/nanoplastics are too common (Turner and Lau 2016).

Novel technologies are studied for PU waste recycling. For instance, biological recycling appears as an eco-friendly solution as it takes place at moderate temperatures, with few energy requirement and with no chemical catalysts. Recently, biological recycling using microbial enzymes as catalysts have been suggested for polyethylene terephthalate (PET) (Gamerith et al. 2017). Thanks to PET depolymerisation, high value monomers such as terephthalic acid can be used as building blocks for further polymer synthesis, in a cradle to cradle approach.

Filamentous fungi which are efficient for the biological degradation of two polyester PU were clearly identified (Magnin et al., 2018). Among them, a strain of *Aspergillus section flavi* and a strain of *Penicillium section Lanata-Divaricata* showed high degrading activities. Identification of degrading enzymes from these fungi is of interest for the development of a biological recycling process. Moreover, studying enzymes could help understanding the complex mechanisms of degradation. Currently, model substrate such as Impranil-DLN (Vega, Main, and Howard 1999; Ruiz and Howard 1999) or pNitrophenylbutyrate (Chen et al. 2008) is needed to locate the enzyme action. Then, an arduous step of enzyme purification is required before sequencing (Nomura et al. 1998).

Exoproteome analysis is a powerful tool to appraise the proteins secreted by a microorganism. The production of extracellular proteins is influenced by the extracellular environment, notably the substrate. Exoproteome of filamentous fungi have been studied to identify enzymes responsible of plant cell wall degradation (Phalip et al. 2005; Kolbusz et al. 2014) . This type of

analysis provides valuable information. For instance, a cellobiohydrolase has been found to act as a sensor for the extracellular environment in *Fusarium graminearum* (Phalip et al. 2005). Incubation of *Pleurotus ostreatus* in the presence of lignocellulosic substrates significantly induced the production of oxydoreductases. This type of enzymes is well-known for the degradation of lignin (Fernandez-Fueyo et al. 2016).

As far as we know, only one publication deals with the exoproteome analysis of a microorganism incubated with synthetic polymers (Wallace et al. 2017). In this publication, all the esterases and lipases secreted by *Pseudomonas pseudoalcaligenes* were listed. Differential analysis revealed that the esterase PpEst is produced only when incubated with a polyester (PEs), the poly(1,4-butylene adipate-co-terephthalate). Cloning and production of this enzyme confirmed activity on this substrate.

In this study, our goal is to perform differential analysis of the exoproteome to identify enzymes that might be responsible for the PU degradation and more specifically the urethane bonds. With this technique, it is possible to work directly on the complex and non-biodegradable polymer. Fungi were incubated with two PEs polyols and with two thermoplastic PU (TPU) synthesized from these PEs. The main difference between the TPU and the PEs is thus the presence of urethane bonds. The goal is to observe potential differences in terms of protein secretion and, possibly, to find enzyme which are overproduced in presence of specific pattern such as the urethane bond.

3. Materials and methods

3.1. Materials

Two PEs polyols were used for both fungal growth and for TPU syntheses: a polycaprolactone (PCL) (Capa 2302 commercialized by Perstorp) and a PEs based on fatty acid dimer (DFA) from rapeseed oil and short diol (Radia 7285 commercialized by Oleon). For the TPU syntheses, the 4,4'-methylene diphenyl diisocyanate (4,4'MDI, Suprasec 1306 - Mn = 250.25 g/mol) was purchased from Huntsman (USA) and the 1,4-butanediol (1,4-BDO, Mn = 90.12 g/mol) was purchased from Invista (USA). The chemical structures of the building blocks composing the PEs and the TPU are available on Figure 4.2.1.

The TPU syntheses were performed into a jacketed reactor at 80°C under nitrogen gas flow. Stoichiometric conditions were used with a NCO/OH ratio of 1, using an equimolar quantity of the PEs polyol and 1,4-BDO. A “one-step” method was performed. The 4,4’-MDI was warmed at 60°C and then introduced into the reactor followed by the mix of the PEs polyol and 1,4-BDO, previously heated at 80°C. Resulting TPU material was then compressed at 145°C with a plate press (Labtech Hot Press) to obtain thin films. They were then cut into pieces of 2 x 2 cm and about 300 mg.

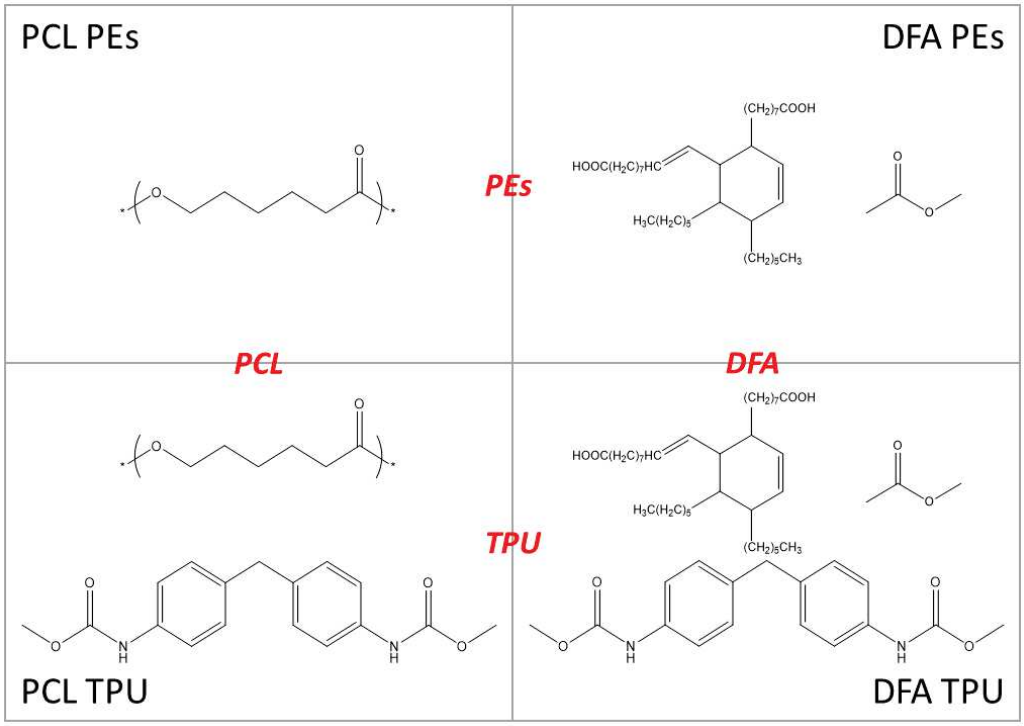


Figure 4.2.1 – Main macromolecular architectures and the corresponding abbreviations. In red, the common pattern between the four polymers.

Minimal medium (MM) was prepared according to Magnin et al. (2018). Buffer with 19 mM of NaH_2PO_4 and 33.5 mM of K_2HPO_4 was supplemented by 15 g/L final of agar and 1.8 mM final of HCl and autoclaved at 121°C for 18 minutes. To 1 L of this buffer was added 10 mL of each of the following solutions, filtered at 0.22 µm with polystyrene filters (in this order): (i) a solution of $MgSO_4$ x $7H_2O$ (250 µM), (ii) a solution containing $FeCl_3x6H_2O$ (147 µM), $ZnCl_2$ (9 µM), $CoCl_2$ (6.5 µM), Na_2MoO_4 x $2H_2O$ (12 µM), $CaCl_2x$ $2H_2O$ (10 µM), $CuCl_2$ x $2H_2O$ (13 µM), $MnCl_2$ x $2H_2O$

(14.6 µM), H_3BO_3 (12 µm) and finally, (ii) a solution of NH_4SO_4 (7.6 mM). Streptomycin at 60 µg/mL final was added in the MM to prevent bacterial growth.

3.2. Fungal growth on polymeric substrates

300 mg of solid TPU or viscous PEs were disposed in 40 mL flasks. It was then rinsed twice with distilled water and once with ethanol 70%. Flasks were allowed to dry overnight at room temperature under a laminar flow hood. Glucose and cellulose solutions at 150 g/L were prepared directly in MM. Glucose solution was filtered at 0.22 µm. Cellulose solution was autoclaved at 121°C for 18 minutes. Cultures of 2 mL of MM with polymers or MM supplemented with glucose or cellulose were inoculated using fungal agar plate culture and an inoculation loop of 1 µL. To determine the incubation time, single cultures were launched with the two fungi on the four different polymer samples at 30°C. Every 2 to 4 days, 30 µL of supernatants were recovered for protein quantity estimation by a Bradford assay. Cultures intended for proteomic analysis were performed with four repetitions. Cultures were stopped and supernatants were recovered and filtered at 0.2 µm.

3.3. Mass spectrometry-based quantitative proteomics

3.3.1. Sample preparation

The supernatants were lysed for 10 seconds with an amplitude of 20%. In order to precipitate and concentrate proteins, 6 volumes of acetone were added to the samples and incubated overnight at -20°C. After a centrifugation step at 8500g for 30 min at 4°C, the supernatants were removed and the pellets were dried for 1 hour under the hood. The pellets were resuspended in 80-100 µL Laemmli buffer (10 mM Tris pH 6.8, 1mM EDTA, 5% β-mercaptoethanol, 5% SDS, 10% glycerol) and shaken for 1h on an agitation wheel. The samples were centrifuged for 1 min at 3000g and the supernatants were kept.

The protein concentration of all samples was determined using the RC-DC protein assay (Bio-Rad) according to the manufacturer's instructions and a standard curve was established using Bovin Serum Albumin (BSA). For each sample, 5 µg of protein lysate were heated at 95°C for 5 minutes and concentrated on a stacking gel by electrophoresis. The gel bands were cut, washed with

ammonium hydrogen carbonate and acetonitrile, reduced and alkylated before trypsin digestion (Promega). The generated peptides were extracted with 60% acetonitrile in 0.1% formic acid followed by a second extraction with 100% acetonitrile. Acetonitrile was evaporated under vacuum and the peptides were resuspended in 10 µL of H_20 and 0.1% formic acid before nanoLC-MS/MS analysis.

3.3.2. NanoLC-MS/MS analysis

NanoLC-MS/MS analyses were performed on a nanoACQUITY Ultra-Performance_LC-system (Waters, Milford, MA) coupled to a Q-Exactive Plus Orbitrap mass spectrometer (ThermoFisher Scientific) equipped with a nanoelectrospray ion source. Samples were loaded into a Symmetry C18 precolumn (0.18 x 20 mm, 5 µm particle size; Waters) over 3 min in 1% solvent B (0.1% formic acid (FA) in acetonitrile) at a flow rate of 5 µL/min followed by reverse-phase separation (ACQUITY UPLC BEH130 C18, 200 mm x 75 µm id, 1.7 µm particle size; Waters) using a binary gradient ranging from 1% and 35% of solvent A (0.1 % FA in H_2O) and solvent B at a flow rate of 450 nL/min. The mass spectrometer was operated in data-dependent acquisition mode by automatically switching between full MS and consecutive MS/MS acquisitions. Survey full scan MS spectra (mass range 300-1800) were acquired in the Orbitrap at a resolution of 70K at 200 m/z with an automatic gain control (AGC) fixed at 3.10^6 ions and a maximal injection time set to 50 ms. The ten most intense peptide ions in each survey scan with a charge state ≥ 2 were selected for MS/MS. MS/MS spectra were acquired at a resolution of 17,5K at 200 m/z, with a fixed first mass at 100 m/z, AGC was set to 1.10^5, and the maximal injection time was set to 100 ms. Peptides were fragmented in the HCD cell by higher-energy collisional dissociation with a normalized collision energy set to 27. Peaks selected for fragmentation were automatically included in a dynamic exclusion list for 60 s. All samples were injected using a randomized injection sequence. To minimize carry-over, a solvent blank injection was performed after each sample. A sample pool comprising equal amounts of all proteins extracts was constituted and regularly injected 4 times during the course of the experiment, as an additional quality control (QC). Protein identification rates and coefficients of variation (CV) monitoring of this QC sample revealed very good stability of the system.

3.3.3. Data interpretation

Raw MS data processing was performed using MaxQuant software (Cox et al. 2014) (v 1.6.0.16). Peak lists were searched against a database including *Aspergillus oryzae RIB40* protein sequences extracted from NCBI (19-04-2018; 20 287 sequences, Taxonomy ID: 510516) or against a database including *Penicillium brasilianum* protein sequences extracted from NCBI (25-04-2018; 11 968 sequences, Taxonomy ID: 104259) using the MSDA software suite (Carapito et al. 2014). MaxQuant parameters were set as follows: MS tolerance set to 20 ppm for the first search and 5 ppm for the main search, MS/MS tolerance set to 40 ppm, maximum number of missed cleavages set to 2, Carbamidomethyl (C) and Oxidation (M) set as variable modifications. False discovery rates (FDR) were estimated based on the number of hits after searching a reverse database and was set to 1% for both peptide spectrum matches (minimum length of seven amino acids) and proteins. Data normalization and protein quantification was performed using the LFQ (label free quantification) option implemented in MaxQuant using a "minimal ratio count" of one. The "Match between runs" option was enabled using a 2 minutes time window after retention time alignment. All other MaxQuant parameters were set as default. To be validated, proteins must be identified in at least three replicates for one condition with a FDR of 1% and with a minimum of three unique peptides in at least one replicate. Differential data analysis was performed using the open-source ProStaR software (Wieczorek et al. 2016). Only proteins with at least three valid values per group as well as the ones "absent" (i.e. 0 valid values) in samples from a given group were kept. A Welch's t-test was applied to compare LFQ intensities of each protein between two conditions. Proteins were considered to be significantly enriched when their Welch's t-test p-values were below 0.05 or when a minimum of 3 unique peptides were validated for the "present/absent" proteins. Venn diagrams were generated[6].

4. Results and discussion

Kinetics of extracellular protein production have been performed prior to proteomic analysis in order to define the time of incubation (Figure 4.1.1). An extracellular protein concentration higher

[6] http://bioinformatics.psb.ugent.be/webtools/Venn/

than 30 mg/L is required. For *A. oryzae*, cultures with the four polymers reached protein concentrations higher than 30 mg/L after 10 days of incubation. Extracellular protein production appeared to be much slower for *P. brasillianum*. After 24 days of incubation, three cultures reached about 30 mg/L of proteins but started also the latent phase. At this time, we decided to stop cultures even if the culture with PCL PEs only reached 15 mg/L of proteins. Cultures in cellulose and glucose were stopped after 5 days of incubation because of the high growth speed.

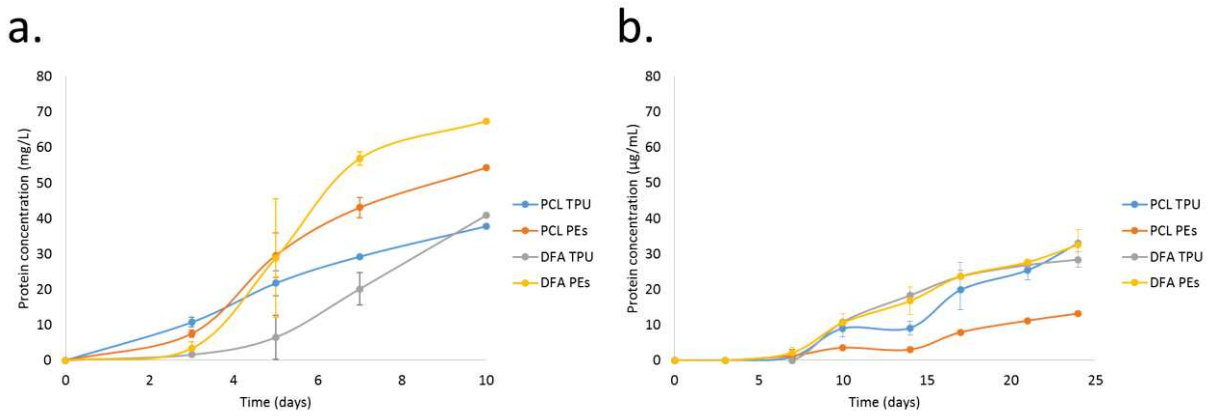


Figure 4.2.2 – Extracellular protein quantification with Bradford method for cultures with (a) Aspergillus oryzae and (b) Penicillium brasilianum

For *A. oryzae*, the dataset is composed of 184 proteins. More proteins were produced by this strain in culture with TPU compared to PEs: 163 and 158 proteins were produced for the PCL TPU and DFA TPU, while 117 and 119 were produced for the PCL PEs and the DFA Pes, respectively. More proteins were produced by the strain of *P. brasilianum.* Contrary to the strain of *A. oryzae*, in this case more proteins were produced in the culture with PEs compared to TPU. The dataset is composed of 503 proteins. 211 and 267 proteins were produced for the PCL TPU and DFA TPU, while 381 and 447 were produced for the PCL PEs and the DFA Pes, respectively. The high number of protein identified might be linked to the extended time of incubation (21 instead of 10 days for *Aspergillus*). Cell lysis might have occurred leading to intracellular protein release in the media.

Venn diagrams aims at evaluating common and different proteins between the four cultures on synthetic polymers based on “present/absent” criteria. To be considered at present in a

condition, a protein must be detected in, at least, 3 of the 4 repetitions and with, at least, 3 unique peptides. A first set of specific enzymes can be selected thanks to Venn diagrams.

Most of the proteins, e.g. 99 of the 184, are common on the four cultures of *A. oryzae* (Figure 4.2.3a). When there is no common molecular patterns, meaning between the PCL TPU and the DFA PEs and between the DFA TPU and the PCL PEs, there is no exclusive common protein (Figure 4.2.3a in red). By contrast, 34 proteins are shared between the cultures made on the two TPU (Figure 4.2.3a in green). These observations are consistent with the assumptions that proteins are produced as a response to a specific polymer pattern (Figure 4.2.3b). Only 161 of the 503 proteins are common between the four cultures of *P. brasilianum*. No clear distribution appeared on the diagram. It has previously been shown that mechanisms of degradation of the DFA TPU and the PCL TPU are highly different and that enzymes responsible of the degradation of the PCL TPU are possibly cell-bound (Magnin et al., 2018). Therefore, it is coherent to have only few common proteins between the DFA TPU and the PCL TPU. Because of the too large number of proteins and the lack of coherence with the cultures of *P. brasilianum*, it was decided to focus only on the strain of *Aspergillus*.

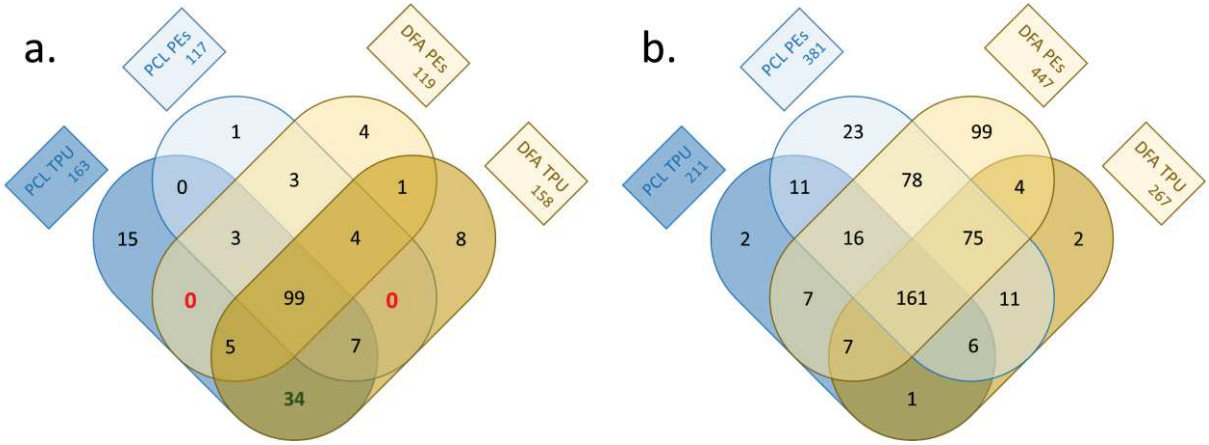


Figure 4.2.3 – Venn diagram for the strain of (a) Aspergillus oryzae and (b) Penicillium brasilianum

The selection of enzymes only relies on the presence or absence of the proteins (Wallace et al. 2017). However, it is important to consider that a protein can be present in two conditions of culture but with a distinct abundances. For this reason, fold-changes (FC) have been studied. This

parameter considers the number of peptides identified for a protein and the intensity of the signals corresponding to these peptides on the MS chromatograms. We focused on proteins with a FC of 10-fold. This parameter was combined with the P-value. Protein presenting p-value higher than 0.05 were removed from the selection. Protein defined as hypothetical protein with no further information in the database were also removed for the selection. Finally, two controls were used to validate the overexpression of proteins. Incubation with glucose allows appraising the basal expression of proteins. Glucose is a negative control as it is assimilated by fungi without enzymatic catalysis required in the extracellular environment (Phalip et al. 2005). Cellulose is used as positive control since it is known to induce enzyme overexpression (Makela et al. 2017; Wallace et al. 2017). Differential analysis with glucose and cellulose was added in the table as present/absent and/or FC value, still with a threshold of 10. Figure 4.2.4 summarizes the data analysis process and the number of protein selected at each step. Before the comparison with the glucose and cellulose controls, 36 enzymes were found to be specific to the TPU pattern (Table 4.2.1) and 4 were specific to the PEs pattern (Table 4.2.2). After the differential analysis with the cellulose and glucose controls (absent or FC < -10), only 21 enzymes remained for the TPU conditions. The proteins Q2U0P3 (amidase) and Q9HGY7 (glyceraldehyde-3-phosphate dehydrogenase) highlighted the importance of comparison with controls. Indeed, level of secretion appeared to be similar with the TPU, glucose and cellulose. It means that these proteins are not overexpressed in contact with TPU but repressed in contact with PEs.

Over the 21 enzymes, 6 are oxydoreductases (EC 1), 14 are hydrolases (EC 3) and one is a lyase (EC 4). Significant increases in the production of oxydoreductases and hydrolases are consistent with the molecular pattern of TPU. Aromatic rings of the 4,4'-MDI might be the target of oxydoreductases while hydrolases might be directed toward urethane bonds. Surprisingly, some of these enzymes are specific of polysaccharides degradation such as the alpha 1,3 glucanase (Q2ULP7) or the beta-glucosidase M (Q2U7H1).

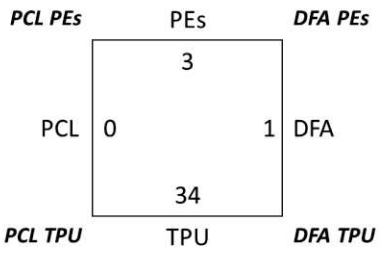
Venn diagrams
« Present/absent » analysis
Protein detected at least 3 times on the 4 samples with 3 unique peptides

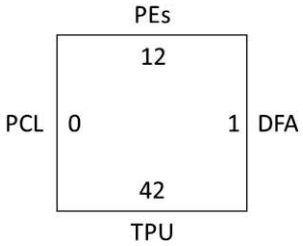
Fold change analysis
FC > 10 or FC < -10

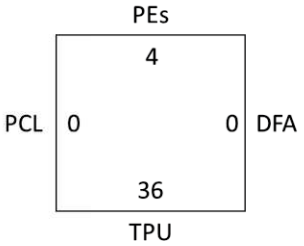
P-value < 0.05
Prot information on database

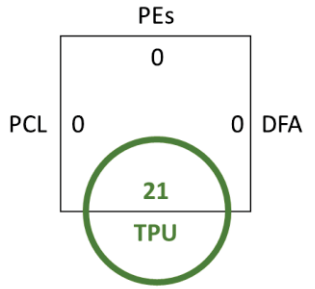
Comparison with glucose/cellulose
Pres/Abs and/or FC < -10

Aspergillus oryzae









Set of specific enzymes for the TPU conditions

Penicillium brasilianum

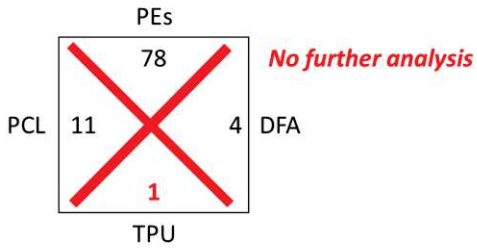


Figure 4.2.4 – Approach developed for enzyme selection

Table 4.2.1 – List of the 36 enzymes overproduced by Aspergillus oryzae incubated with the two TPU by comparison to the PEs

Entry	SwissProt	EC number	FC PCL PEs/ PCL TPU	FC DFA PEs/ DFA TPU	Glucose Abs/Pres	FC Glu/PCL	FC Glu/DFA	Cellulose Abs/Pres	FC Cell/PCL	FC Cell/DFA	TPU specific
gi|121796738|sp|Q2TW34.1 RecName: Full=Catalase-peroxidase; Short=CP; AltName: Full=Peroxidase/catalase	Q2TW34	1.11.1.21			Abs			Abs			X
gi|121797915|sp|Q2TZQ9.1 RecName: Full=Probable glucan endo-1,6-beta-glucosidase B; AltName: Full=Beta-1,6-glucanase B; AltName: Full=Endo-1,6-beta-D-glucanase B; AltName: Full=Endo-1,6-beta-glucanase B; Flags: Precursor	Q2TZQ9	3.2.1.75			Abs			Pres	-2.24	-1.33	
gi|121798552|sp|Q2U1X8.1 RecName: Full=Probable arabinan endo-1,5-alpha-L-arabinosidase C; AltName: Full=Endo-1,5-alpha-L-arabinanase C; Short=ABN C; Flags: Precursor	Q2U1X8	3.2.1.99			Pres	-2.76	2.12	Pres	5.99	34.96	
gi|121798879|sp|Q2U325.1 RecName: Full=Probable beta-glucosidase G; AltName: Full=Beta-D-glucoside glucohydrolase G; AltName: Full=Cellobiase G; AltName: Full=Gentiobiase G; Flags: Precursor	Q2U325	3.2.1.21			Abs			Abs			X
gi|121801892|sp|Q2UDK7.1 RecName: Full=Probable beta-glucosidase M; AltName: Full=Beta-D-glucoside glucohydrolase M; AltName: Full=Cellobiase M; AltName: Full=Gentiobiase M; Flags: Precursor	Q2UDK7	3.2.1.21	36.95	20.99	Abs			Pres	-280.92	-92.15	X
gi|121804190|sp|Q2ULM2.1 RecName: Full=probable leucine aminopeptidase 2; AltName: Full=Aminopeptidase II; AltName: Full=Leucyl aminopeptidase 2; Short=LAP2; Flags: Precursor	Q2ULM2 (Blinkovsky et al. 2000)	3.4.11	77.83	76.76	Pres	-26.89	-23.23	Pres	-23.99	-20.72	X
gi|121804405|sp|Q2UMC1.1 RecName: Full=Probable carboxypeptidase AO090003000058; AltName: Full=Peptidase M20 domain-containing protein AO090003000058; Flags: Precursor	Q2UMC1	3.4.17			Abs			Abs			X
gi|121938381|sp|Q2U8R6.1 RecName: Full=Probable pectate lyase A; Flags: Precursor	Q2U8R6	4.2.2.2			Abs			Pres	1.68	9.48	
gi|169762856|ref|XP_001727328.1 L-xylulose reductase	Q2UD76	1.1.1.10			Abs			Abs			X
gi|169763026|ref|XP_001727413.1 beta-galactosidase	Q2UCZ1	3.2.1.23			Abs			Abs			X
gi|169763584|ref|XP_001727692.1 extracellular phytase	Q2UC62	3.1.3.1			Abs			Abs			X
gi|169764221|ref|XP_001816582.1 class II aldolase/adducin domain protein	Q2UV35	4.1.2.13			Abs			Abs			X
gi|169766420|ref|XP_001817681.1 N-acetylglucosaminidase	Q2URY6	3.2.1.52	888.82	172.19	Pres	-963.9	-320.26	Abs			
gi|169766880|ref|XP_001817911.1 enoyl-CoA hydratase/isomerase family protein	Q2URA6	4.2.1.17			Abs			Abs			
gi|169767820|ref|XP_001818381.1 alpha-mannosidase	Q2UPY6	3.2.1			Abs			Abs			X
gi|169768396|ref|XP_001818668.1 glycerophosphoryl diester phosphodiesterase family protein	Q2UP49	3.1.4.46			Abs			Pres	-3.13	-1.21	
gi|169770101|ref|XP_001819520.1 alpha 1,3 glucanase, GH71 family	Q2ULP7	3.2.1.59	28.95	55.91	Abs			Abs			X

Entry	SwissProt	EC number	FC PCL PEs/ PCL TPU	FC DFA PEs/ DFA TPU	Glucose Abs/Pres	FC Glu/PCL	FC Glu/DFA	Cellulose Abs/Pres	FC Cell/PCL	FC Cell/DFA	TPU specific
gi|169771733|ref|XP_001820336.1 rhamnogalacturonan acetylesterase	Q2UJD1	3.1.1.86			Abs			Pres	14.23	16.52	
gi|169771773|ref|XP_001820356.1 alpha-L-rhamnosidase A	Q2UJB1	3.2.1.40			Abs			Abs			X
gi|169772357|ref|XP_001820647.1 FAD binding domain protein	Q2UIH0	1			Pres	1.07	1.14	Abs			
gi|169774545|ref|XP_001821740.1 FAD/FMN-containing isoamyl alcohol oxidase MreA-like protein	Q2UFC7	1			Abs			Abs			X
gi|169778323|ref|XP_001823627.1 beta-glucosidase M	Q2U7H1	3.2.1.21			Abs			Abs			X
gi|169778633|ref|XP_001823781.1 carboxypeptidase 2	Q2U717	3.4.16-18			Abs			Abs			X
gi|169779089|ref|XP_001824009.1 repressible alkaline phosphatase	Q2U6D9	3.1.3.1			Abs			Pres	-20.87	-16.07	
gi|169783086|ref|XP_001826005.1 amidase	Q2U0P3	3.5.1.4			Pres	-1.08	-2.69	Pres	-1.59	-3.95	
gi|169783572|ref|XP_001826248.1 oxidoreductase, 2-nitropropane dioxygenase family	Q2U000	1.13.11.32	33.20	46.36	Abs			Abs			X
gi|30580402|sp|Q9HGY7.1 RecName: Full=Glyceraldehyde-3-phosphate dehydrogenase; Short=GAPDH ;gi|83768244|dbj|BAE58383.1 unnamed protein product	Q9HGY7 (Nakajima et al. 2000)	1.2.1.12			Pres	-1.86	1.16	Pres	-2.51	-1.17	
gi|317148402|ref|XP_001822749.2 vacuolar aspartyl aminopeptidase Lap4 ; gi|83771484|dbj|BAE61616.1 unnamed protein product	Q2U9Z9	3.4.11.21	45.23	230.54	Pres	-15.24	-4.73	Pres	-71.71	-22.26	
gi|317155313|ref|XP_001825009.2 thioredoxin reductase GliT ; gi|83773751|dbj|BAE63876.1 unnamed protein product	Q2U3I9	1.8.1.9			Abs			Pres	-7.7	-38.09	
gi|46395587|sp|Q7Z9L3.1 RecName: Full=Glucan 1,3-beta-glucosidase A; AltName: Full=Exo-1,3-beta-glucanase 1; AltName: Full=Exo-1,3-beta-glucanase A; Flags: Precursor; gi|317144408|ref|XP_001820101.2 glucan 1,3-beta-glucosidase A	Q7Z9L3 (Tamano et al. 2007)	3.2.1.58	95.36	42.20	Pres	-673.57	-235.32	Pres	-56.95	-19.9	X
gi|83767900|dbj|BAE58039.1 unnamed protein product ; gi|317145126|ref|XP_001820041.2 endo-1,3-beta-glucanase Engl1	Q2UK76	3.2.1.39			Abs			Pres	1.05	2.91	
gi|83767943|dbj|BAE58082.1 unnamed protein product ; gi|317144383|ref|XP_001820084.2 glutamate carboxypeptidase	Q2UK33	3.4.17.11			Abs			Abs			X
gi|83768503|dbj|BAE58640.1 unnamed protein product ; gi|317145271|ref|XP_001820642.2 hydrolase	Q2UIH5	3			Abs			Abs			X
gi|83771436|dbj|BAE61568.1 unnamed protein product ; gi|317148342|ref|XP_001822701.2 conidial pigment biosynthesis oxidase Arb2/brown2	Q2UA47	1			Pres	-773.66	-52.88	Abs			X
gi|83771931|dbj|BAE62061.1 unnamed protein product ; gi|317149833|ref|XP_001823194.2 FAD-dependent oxygenase	Q2U8Q4	1			Abs			Pres	-223.11	-47.87	X
gi|94730375|sp|Q9Y8E3.2 RecName: Full=Dipeptidyl-peptidase 5; AltName: Full=Alanyl dipeptidyl peptidase; AltName:	Q9Y8E3	3.4.14			Pres	61.08	32.75	Abs			

Table 4.2.2 – List of the 4 enzymes overproduced by Aspergillus oryzae incubated with the two PEs by comparison to the TPU

Entry	SwissProt	EC number	FC PCL PEs/ PCL TPU	FC DFA PEs/ DFA TPU	Glucose Abs/Pres	FC Glu/PCL	FC Glu/DFA	Cellulose Abs/Pres	FC Cell/PCL	FC Cell/DFA
gi|169762550|ref|XP_001727175.1 aminopeptidase Y [Aspergillus oryzae RIB40]	Q2UDM9	3.4	-18.71	-23.74	Abs			Pres	-6.82	-9.24
gi|169777249|ref|XP_001823090.1 carboxypeptidase S1 [Aspergillus oryzae RIB40]	Q2U908	3.4.16			Pres	153.32	87.86	Pres	3.21	1.84
gi|169777981|ref|XP_001823456.1 alpha-xylosidase [Aspergillus oryzae RIB40]	Q2U7Z2	3.2.1.20			Pres	-58.06	-138.23	Pres	-4.99	-11.89
gi|169782882|ref|XP_001825903.1 tripeptidyl-peptidase sed2 [Aspergillus oryzae RIB40]	Q2U0Z5	3.4	-21.42	-47.31	Pres	1.67	1.83	Pres	-4.57	-4.17

In the literature, reported enzymes showing catalytic activity on the urethane bond are proteases (Campinez et al. 2013; Marchant et al. 1987) or amidases (Gamerith et al. 2016). Among our set of 21 enzymes, 4 correspond to these classes: the carboxypeptidase (Q2UMC1), the carboxypeptidase 2 (Q2U717), the glutamate carboxypeptidase (Q2UK33) and the leucine aminopeptidase 2 (Q2ULM2). Besides, the activity of the last one has already been characterized (Blinkovsky et al. 2000). Activity assays on peptides and pNitroanilides showed that this enzyme is active on a broad spectrum of substrates thus comforting the hypothesis that it may act on the urethane bond.

5. Conclusion

Differential analysis of protein secretion by *A. oryzae* and *P. brasilianum* grown on four synthetic and non-biodegradable polymers was performed. Because of the difference of degradation mechanisms between the two TPU, *P. brasilianum* appeared to be unsuitable for this study. The dataset of *A. oryzae* is composed of 184 proteins. After a stringent process of differential protein selection, 21 enzymes, mainly hydrolases and oxydoreductases appeared to be specific of the TPU substrates by comparison to PEs, glucose and cellulose.

Finally, four peptidases have been identified as good candidates for the cleavage of the urethane bond. Activity assays with these enzymes on appropriate substrates should be performed to confirm this hypothesis and to validate the present exoproteomic approach.

This study offers an efficient solution for the discovery and characterization of PU-degrading enzymes. Development of enzymatic recycling process using enzymes discovered that way could address the major environmental challenge of the plastic pollution in the coming years.

Acknowledgements

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Conclusion

Le chapitre 4 de cette thèse propose une méthode d'identification d'enzymes microbiennes de dégradation de polyuréthanes en relation avec les résultats obtenu dans le chapitre 3. Cette méthode a pour avantage d'être directement applicable à un substrat polymère récalcitrant et de ne pas nécessiter la purification de l'enzyme d'intérêt.

L'analyse de l'exoprotéome d'*Aspergillus oryzae* a pu être menée avec succès en proposant quatre peptidases potentiellement actives sur la partie uréthane des polyesters PU. Cependant, afin de valider cette démarche, il est nécessaire d'approfondir l'étude de ces 4 enzymes. Pour cela, il conviendrait notamment de cloner les enzymes dans un châssis de levure afin de les produire en grande quantité. L'activité de dégradation de ces enzymes pourrait ainsi être testée à l'aide des méthodes mises au point et décrites dans le chapitre 2 (hydrolyse d'un substrat uréthane modèle, analyse de TPU dégradés par microscopie et chromatographie d'exclusion stérique, analyse de produits de la dégradation de TPU).

Conclusion Générale

L'objectif de cette thèse était d'identifier et caractériser des enzymes et microorganismes capables de dégrader les polyuréthanes (PU), afin de proposer des voies de recyclage par voie biologique. Il s'agit de contrôler et valoriser la fin de vie de ces matériaux dans une approche de développement durable avec des approches respectueuses de l'environnement.

Ces travaux de thèse s'intègrent aussi dans le cadre du projet européen Horizon 2020 « P4SB » (*From Plastic waste to Plastic value using Pseudomonas putida Synthetic Biology*). Aussi ces travaux ont été développés en concertation et partenariat avec des partenaires académiques et industriels (Soprema, Proteus).

Après une introduction générale, ce mémoire de thèse est composé de quatre chapitres. Dans un **premier chapitre**, une synthèse bibliographique exhaustive a permis de mettre en exergue le potentiel d'enzymes et de microorganismes capable de dégrader des PU avec des structures chimiques complexes et variées. Les techniques analytiques proposées pour évaluer cette dégradation ont été plus particulièrement étudiées. Un grand nombre de ces techniques, telles que la chromatographie d'exclusion stérique (SEC), la spectroscopie infrarouge ou encore l'analyse des produits de dégradation par spectrométrie de masse, a ensuite pu être déployé dans cette étude et se trouve décrit au cours des chapitres suivants. Le premier chapitre a permis de définir le concept de substrat modèle, indispensable au développement de criblages enzymatiques.

Les chapitres 2, 3 et 4 constituent la partie expérimentale de ce mémoire. Le **deuxième chapitre** décrit le criblage et la caractérisation d'enzymes de dégradation des PU. Deux enzymes aux activités complémentaires ont ainsi été sélectionnées : E3576, une estérase, active sur les segments polyesters d'un polyester PU et E4143, une amidase, active sur la liaison uréthane de molécules de faibles masses molaires. Ces expériences constituent une étude complète. Dans un premier temps, la dégradation enzymatique a été caractérisée. L'apparition d'oligomères a par exemple été suivie par SEC. Les produits de dégradation ont ensuite été identifiés par spectroscopie de masse et RMN. Ces composés possédant des groupements fonctionnels actifs du type acide carboxylique, hydroxyle ou amine pourraient donc être valorisés, par exemple au

travers de la synthèse d'une seconde génération de polymères du type polyamide ou polyuréthane.

La voie microbienne de dégradation des PU a ensuite été explorée et investiguée. Les résultats de cette étude sont présentés dans le **troisième chapitre**. Trois champignons ont démontré une capacité à dégrader deux polyesters PU. Les trois souches actives ont été identifiées comme étant *Alternaria sp*., *Aspergillus oryzae* et *Penicillium brasilianum*. Les mécanismes et le rendement de dégradation du PU dont le polyester polyol est obtenu à partir de dimère d'acides gras sont similaires pour les trois souches. La souche de *Penicillium* a montré une efficacité supérieure aux deux autres champignons pour la dégradation du PU à base de polycaprolactone (PCL). Les analyses montrent un « sillon » à la surface du polymère correspondant possiblement aux filaments fongiques. Couplé à des mesures d'activité enzymatique très faibles du surnageants, ces analyses suggèrent l'implication d'enzymes pariétales pour cette dégradation.

Dans une perspective de recyclage par voie biologique, nous avons tenté d'identifier les enzymes responsables de la dégradation des polyesters PU chez *A. oryzae* et *P. brasilianum* via une analyse protéomique différentielle. Ces expériences constituent le dernier et **quatrième chapitre** de ce mémoire. Cette analyse n'a pas pu être menée à son terme pour *P. brasilianum* car le nombre de protéines s'est avéré trop important et aucune tendance claire n'a pu être dégagée parmi les protéines communes aux cultures sur les différents substrats. Cela confirme toutefois les résultats du chapitre précédent à savoir que les mécanismes de dégradation du PU à base de dimère d'acide gras et du PU à base de PCL sont différents. Le premier fait intervenir des enzymes extracellulaires contrairement au second qui mobilise des enzymes pariétales. En revanche l'analyse différentielle de l'exoprotéome d'*Aspergillus oryzae* a conduit à des résultats particulièrement intéressants. La souche d'*A. oryzae* produit ainsi plus d'hydrolases et d'oxydoréductases lorsque le milieu de culture contient les polyesters PU que lorsqu'il ne repose que sur les polyesters polyols correspondant. Quatre peptidases surproduites par *A. oryzae* incubé avec les deux TPU s'avèrent être de bons candidats pour la dégradation de la liaison uréthane de ces polymères.

Nous avons donc abordé la dégradation biologique des polyuréthanes sous une perspective assez large. Les réactions de dégradation réalisées font intervenir aussi bien des enzymes que des microorganismes et ceci sur un panel varié de substrats uréthanes. Des avancées majeures telles que la combinaison d'enzymes aux activités complémentaires et la découverte de champignons avec une forte activité de dégradation ont été présentées. Contrairement à la grande majorité des études menées sur cette problématique, nous avons porté une attention toute particulière à la démonstration du clivage de la liaison uréthane. Ce groupe chimique est connu pour être particulièrement résistant à l'hydrolyse et au vieillissement, faisant ainsi des matériaux à très longue durée de vie et persistants. Un travail et un effort important ont été développés pour à la fois comprendre les mécanismes de dégradation et démontrer la faisabilité du recyclage biologique des PU.

Les rendements de dégradation d'un PU à base de PCL sont de 30% de perte de masse après 50 jours pour la dégradation enzymatique et de moins de 10% après 60 jours pour la dégradation fongique. Ces rendements, bien que significatifs et élevés au regard de la littérature existante, doivent être encore améliorés mais ouvrent d'ores et déjà la voie vers de nouvelles perspectives de développement pour un recyclage efficace des polyuréthanes.

Perspectives

A l'issue de ces travaux de thèse, nous disposons donc de deux enzymes et trois champignons dégradant les polyuréthanes (PU). Le chapitre 4 propose quatre enzymes candidates à la dégradation de la liaison uréthane. Les gènes codants pour ces enzymes vont être intégrés dans une souche de levure permettant ainsi une production hétérologue. L'activité des enzymes ainsi produites va pouvoir être évaluée par les méthodes proposées dans le deuxième chapitre de la thèse telle que l'hydrolyse du substrat uréthane de faible masse molaire. Ainsi le panel d'enzymes à disposition pourrait être augmenté.

Nous pouvons alors définir deux perspectives principales. La première consiste à optimiser les systèmes jusqu'à l'obtention de rendements de dégradation suffisamment élevés. La seconde est de continuer à explorer les substrats uréthanes pouvant être dégradés par les microorganismes et enzymes à disposition. L'optimisation des conditions de dégradation (température, pH, oxygénation…) sera un préalable pour poursuivre l'étude. L'objectif sera de dégrader d'autres types de PU avec différentes architectures macromoléculaires (mousses,…) et de cibler ainsi l'ensemble des PU produits industriellement qui sont donc aussi des sources de déchets. Ces deux principaux axes sont abordés ci-dessous.

Au-delà des pistes de travail proposées ci-dessus, des premières expériences ont été réalisées en fin de thèse pour initier un nouveau volet expérimental.

Optimisation des réactions de dégradation

Un grand nombre de paramètres peuvent être modifiés, aussi bien pour les enzymes que les microorganismes, afin d'améliorer les rendements de dégradation. Les paramètres de réactions tels que la température, le pH ou la vitesse d'agitation sont à optimiser.

Pour la dégradation microbienne, l'oxygénation est capitale. En effet, la consommation d'oxygène est indispensable à la croissance d'organismes aérobies. La composition du milieu de culture influe également sur la croissance et sur la production d'enzymes par les micro-organismes. Des premiers essais sur l'optimisation du milieu de culture ont été réalisés. La majorité des publications traitant de la dégradation fongiques des PU implique une source de carbone autre que le polymère à dégrader (Alvarez-Barragan et al. 2016; Khan et al. 2017; Oprea et al. 2018).

Dans le sous-chapitre 3.1, nous avons montré que le milieu PDB est un frein à la dégradation. En effet, les champignons consomment préférentiellement les substrats du PDB présents en abondance, simples (glucose) ou proches des substrats présents dans les écosystèmes fongiques (amidon), et donc ne nécessitant pas d'apports énergétiques conséquents. Pour cette expérience d'optimisation des milieux de culture, l'idée est d'utiliser ces co-substrats à des concentrations plus faibles, ou d'autres substrats en substitution. La littérature fait ainsi état de plusieurs co-substrats d'intérêt pour accompagner et favoriser la dégradation de polymères synthétiques : le glucose (Hadibarata and Kristanti 2012), le sucrose (Khan et al. 2017), le glycérol (Khan et al. 2017), PDB (Alvarez-Barragan et al. 2016), un surfactant : le Tween 80 (Novotný et al. 2004) et l'Impranil-DLN® (Cosgrove et al. 2010).

Ces substrats permettent de promouvoir la croissance du champignon pour accroître la biomasse. Quand la biomasse est en quantité suffisante, l'arsenal enzymatique nécessaire pour dégrader le polymère peut être déployé en présence de celui-ci. Il est également possible que le co-substrat possède un motif commun avec le polymère récalcitrant. Le co-substrat simple induit la production d'enzyme reconnaissant ce motif. Ces enzymes vont ensuite être à-même d'agir à la fois sur le co-substrat et le polymère. C'est par exemple le cas du Tween 80 possédant une liaison ester et de l'Impranil possédant des liaisons ester et uréthane. La peptone, un hydrolysat protéique, a été ajoutée à la liste des co-substrats. En effet, elle peut induire la production de protéases à large spectre, ayant potentiellement une activité sur la liaison uréthane.

Les expériences ont été réalisées avec les trois souches caractérisées dans le sous-chapitre 3.2 : *Alternaria*, *Penicillium* et *Aspergillus*, sur le TPU base PCL. Tous les co-substrats ont été ajoutés directement dans le MM à une concentration de 2 g/L. Les cultures ont été stoppées après 3 semaines d'incubation à 30°C. Les morceaux de TPU ont été lavés et pesés pour évaluer la perte de masse (Figure P5a). Les surnageants de cultures ont été prélevés afin de tester l'activité estérase sur le substrat modèle pNitrophénylacétate (pNPA) (Figure P5b). Pour évaluer l'impact des co-substrats, il convient donc de comparer les pertes de masse et l'activité estérase en présence de co-substrats avec l'incubation en MM.

Comme observé précédemment, la souche de *Penicillium* est celle qui engendre la perte de masse la plus importante sur le TPU à base de PCL. En comparaison de l'incubation en MM seul, aucun des co-substrats proposés dans cette étude ne permet l'amélioration des rendements de dégradation. La plupart ont même tendance à inhiber cette dégradation. En revanche, certains co-substrats stimulent la production d'estérases mais celles-ci semblent ne pas avoir d'impact sur la dégradation. Au regard des milieux testés, le MM seul serait donc optimal pour la dégradation du TPU base PCL.

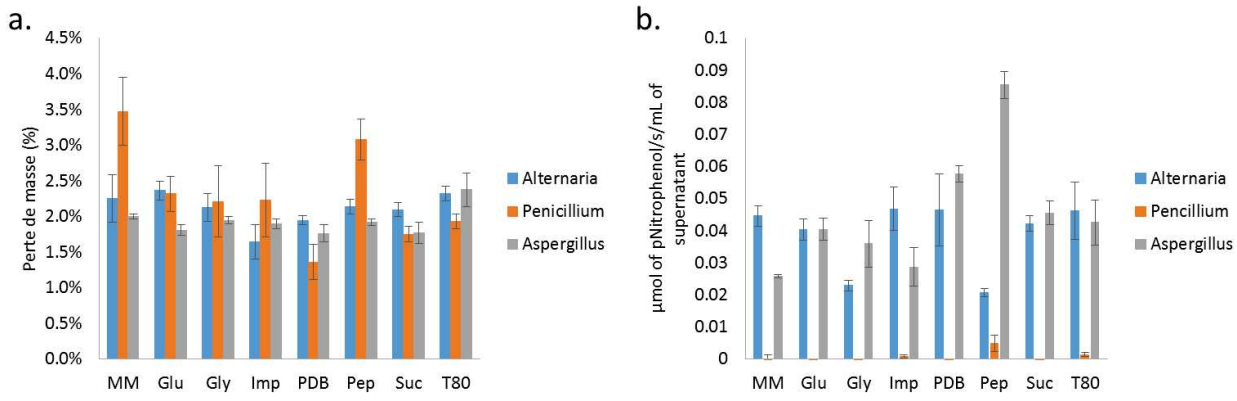

Figure P5 – Influence des co-substrats sur (a) la perte de masse et (b) l'activité estérase du surnageant pour des fragments de TPU base PCL incubés 3 semaines avec 3 différentes souches de champignons

Pour la dégradation enzymatique, les premières étapes d'optimisation envisagées sont les variations de la température et du pH. Par la suite, l'immobilisation des enzymes pourrait être envisagée dans le but d'augmenter leur durée d'utilisation. L'immobilisation peut se faire par adsorption sur un support inerte, par piégeage dans un gel ou encore par microencapsulation.

Etude des capacités de degradation des enzymes et champignons sur des substrats PU plus complexes

Les enzymes et champignons d'intérêt identifiés et décrits au cours de cette thèse présentent des capacités de dégradation sur des substrats uréthanes modèles de faible masse molaire et sur des polyesters TPU. Les polyéthers TPU ont, quant à eux, montrés une résistance très forte à la

dégradation biologique. En effet, il est connu que la fonction éther est plus résistante que l'ester aux phénomènes de dégradation. Il conviendra donc de tester de nouveau la dégradation de ces polymères récalcitrants dans des conditions optimales.

Par ailleurs, les mousses PU représentent la grande majorité (environ 68%) des PU (Pillai et al. 2016) et sont donc la principale source de déchets PU. Le recyclage de celles-ci est donc un enjeu environnemental d'envergure. Ceci d'autant plus que, de faible densité, les mousses PU sont facilement dispersées dans la nature. Elles sont retrouvées en grande quantité dans les micro-plastiques qui polluent les océans. De plus, ces mousses sont constituées de PU thermodurcissables (chaînes réticulées) et ne peuvent donc pas être revalorisées par chauffage (recyclage matière) comme les thermoplastiques. Les procédés actuels de recyclage des mousses de PU génèrent des produits à faible valeur ajoutée à l'image de l'utilisation de mousses broyées qui sont ajoutés dans des matrices cimentaires ou bitumineuses, ou dans la production de tapis[7]. Les synthons obtenues par recyclage biologique pourraient avoir une valeur ajoutée supérieure.

Finalement, avec l'optimisation des procédés de dégradation et l'élargissement de la gamme de PU pouvant être dégradés, une suite à ces travaux porterait sur le changement d'échelle du procédé ainsi que la récupération et la valorisation des produits de dégradation avec pour objectif final l'obtention d'un procédé de recyclage industrialisable.

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Audrey MAGNIN

Dégradation de polyuréthanes thermoplastiques. par voies enzymatique et microbienne. Vers un recyclage biologique et durable.

Résumé

Les polyuréthanes (PU) sont des polymères synthétiques majoritairement destinés à des applications long-termes, à l'image des panneaux d'isolation pour le secteur de la construction. La gestion des déchets PU est un enjeu majeur pour limiter l'impact environnemental de ces matériaux. Parmi les solutions envisageables, le recyclage biologique, qui repose sur la dépolymérisation enzymatique des PU, présente un intérêt majeur en permettant une valorisation des synthons issus de cette dégradation. L'objectif de ces travaux de thèse était d'identifier des enzymes et champignons capables de dégrader les PU. Deux enzymes aux activités de dégradation complémentaires ont démontré une capacité à dépolymériser efficacement un polyester PU thermoplastique. Les produits de cette dégradation ont pu être caractérisés. Trois souches de champignons dégradant deux polyesters PU ont été isolées à partir de prélèvements environnementaux. Une analyse protéomique met en évidence quatre enzymes produites par la souche d'*Aspergillus oryzae* pouvant potentiellement cliver la liaison uréthane. Au-delà de proposer de nouvelles entités biologiques dégradant les PU, ces travaux décrivent également le développement d'un panel de méthodes d'analyses pertinentes de la dégradation biologique PU.

Mots clés : Recyclage biologique, polyuréthanes, dégradation fongique, catalyse enzymatique, protéomique

Abstract

Polyurethanes (PU) are synthetic polymers mainly intended for long-term uses such as isolation panels for buildings applications. PU waste management is a major challenge to limit the environmental impact of these materials. Among the possible solutions, biological recycling, consisting in the enzymatic depolymerization of polymers is of major interest since it allows for the recovery of valuable building-blocks. The purpose of this work was thus to identify and characterize enzymes and fungi able to degrade PU. Two enzymes with complementary degradation activities display abilities to efficiently depolymerize a polyester PU. Resulting degradation products were characterized. Three strains of fungi, isolated from environment, were found to degrade two polyester PU. A proteomic analysis highlights four enzymes produced by *Aspergillus oryzae* potentially able to cleave the urethane bond. Besides reporting new biological entities for the PU degradation, this work also describes the development of a set of analytical tools for the appropriate analysis of the biological degradation of PU.

Keywords: Biological recycling, polyurethanes, fungal degradation, enzymatic catalysis, proteomic