

**Ing. Lucie Husárová**

**Studium biodegradace polymerních materiálů s využitím  
kombinace konvenčních a molekulárně biologických metod**

**Study of polymer material biodegradation by combination of conventional  
and molecular biology methods**

**Disertační práce**

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Školitel:	doc. Mgr. Marek Koutný, Ph.D	
Konzultant:	doc. RNDr. Leona Buňková, Ph.D	

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## **ABSTRAKT**

Cílem disertační práce bylo studium vybraných polymerních materiálů z pohledu jejich schopnosti podléhat biodegradaci. Biodegradace byly studovány metodami založenými na materiálové charakterizaci polymerů, produkci plynů během procesu biodegradace a byly zkoumány možnosti použití molekulárně biologických metod. Sledován byl rozsah biodegradability připravených vzorků polyethylenů s různými prooxidanty, které byly podrobeny abiotické oxidaci. Biodegradace byly uskutečňovány v prostředí půdy a kompostu. Podrobnější výzkum byl věnován zavedení vybraných molekulárně biologických metod v laboratoři fakulty, zvláště technice gelové elektroforéze v teplotním gradientu (TGGE). S touto separační technikou byly optimalizovány související postupy jako izolace nukleových kyselin, polymerázová řetězová reakce (PCR), vyhodnocování TGGE gelů, klonování a sekvenování. Sledována byla také biodegradace polyvinylalkoholu (PVA) za denitrifikačních podmínek v prostředí aktivovaného kalu. Rovněž byla zkoumána dynamika a složení mikrobiálního společenstva účastnícího se biodegradace kyseliny polymléčné (PLA) v závislosti na její formě a distribuci molekulových hmotností. Výzkumy byly prováděny jak v prostředí půdy, tak kompostu. V uvedených experimentech byly aplikovány zvolené molekulárně biologické metody, abychom identifikovali mikroorganismy zodpovědné za rozklad polymeru.

**Klíčová slova:** PE, PVA, PLA, prooxidanty, izolace DNA, PCR, TGGE, sekvenování, SEM



## **ABSTRACT**

The thesis studied selected polymeric materials and their biodegradation using methods based on material characterization of polymers, gas production during biodegradation process and selected molecular biology methods. Biodegradability of prepared polyethylene samples with various prooxidants previously subjected to abiotic oxidation were studied in the environment of soil and compost. Further research was focused on the introduction of selected molecular biology methods in the laboratory of the faculty, especially gel electrophoresis in the temperature gradient (TGGE). This separation technique was optimized along with related procedures such as nucleic acid isolation, polymerase chain reaction (PCR), evaluation of TGGE gels, cloning and preparation of DNA samples for sequencing. Biodegradation of polyvinyl alcohol (PVA) under denitrifying conditions in an activated sludge was also investigated subsequently with the dynamics and composition of the microbial communities involved in the biodegradation of polylactic acid (PLA), depending on its form and the molecular weight distribution. These experiments were conducted in the compost. In these experiments, the selected molecular methods were applied to identify the microorganisms responsible for the degradation of polymers.

## OBSAH

ABSTRAKT .....	2
ABSTRACT .....	3
OBSAH.....	4
1. SOUČASNÝ STAV ŘEŠENÉ PROBLEMATIKY.....	5
1.1. Problematika odpadů obsahujících polymerní materiály .....	5
1.2. Biodegradace polymerních materiálů.....	6
1.2.1. Enzymatická biodegradace.....	7
1.3. Vybrané typy biodegradabilních polymerů.....	9
1.3.1. Polyethylen (PE) s prooxidanty.....	9
1.3.2. Polyvinylalkohol (PVA).....	9
1.3.3. Polyestery (PES) a kopolyestery .....	10
2. METODY STUDIA BIODEGRADACE POLYMERŮ.....	11
2.1. Metody založené na materiálové charakterizaci polymerů .....	11
2.2. Metody založené na produkci plynů.....	11
2.3. Možnosti použití molekulárně biologických metod .....	12
2.3.1. Izolace DNA .....	12
2.3.2. TGGE .....	13
2.3.3. Fylogenetická analýza .....	15
2.3.4. Tvorba a sekvenace DNA knihoven.....	16
2.3.5. Real- time PCR (RT-PCR).....	16
3. CÍLE PRÁCE .....	17
4. ZVOLENÉ METODY ZPRACOVÁNÍ.....	18
4.1. Stanovení míry biodegradace .....	18
4.1.1. Úprava vzorku pro biodegradační testy.....	18
4.1.2. Biodegradační testy .....	18
4.2. Stanovení míry biodegradace PVA ve vodném prostředí .....	19
4.3. Molekulárně biologické metody.....	19
4.3.1. Izolace nukleových kyselin .....	19
4.3.2. PCR.....	19
4.3.3. TGGE .....	19
4.3.4. Excise a purifikace bandů.....	20
4.3.5. Klonování a tvorba DNA knihovny .....	20
4.3.6. Real-time PCR (RT-PCR) .....	20
5. PUBLIKOVANÉ PRÁCE.....	21
5.1. Srovnání biodegradability různých PE fólií obsahující prooxidanty (Příloha P1) ...	21
5.2. Aerobní biodegradace polyethylenových fólií obsahující prooxidanty a uhličitan vápenatý jako plnivo (Příloha P2).....	22
5.3. Biodegradace PVA za denitrifikačních podmínek (Příloha P3).....	22
5.4. Vliv molekulové hmotnosti a formy PLA na její biodegradaci (Příloha P4) .....	23
6. ZÁVĚR.....	25
7. CURRICULUM VITAE .....	27
8. SEZNAM AKTIVIT A PUBLIKACÍ .....	28
9. LITERATURA .....	30
10. SEZNAM OBRÁZKŮ .....	35
11. SEZNAM PŘÍLOH .....	36

# 1. SOUČASNÝ STAV ŘEŠENÉ PROBLEMATIKY

## 1.1. Problematika odpadů obsahujících polymerní materiály

Problém neustále se zvyšujícího množství plastového odpadu se snaží vědci řešit již několik let. Důvod, proč stále nedochází k redukci tohoto množství, představuje pokrok v moderních technologiích, které činí ze syntetických polymerů především cenově dostupný a tedy upřednostňovaný materiál. A tak se skládky odpadů dále plní výrobky na jedno použití, které vykazují vysokou odolnost vůči degradaci za nízkou pořizovací cenu. Chceme-li tedy plastový odpad, jakožto všudypřítomnou součást našeho životního prostředí, redukovat, řešením je hledání alternativ, např. použití biodegradabilních materiálů. Jejich výhodou by mohla být nejen jejich rozložitelnost pomocí mikroorganismů, jsou-li vneseny do půdy, ale také možnost kompostovat tyto materiály spolu s ostatními složkami biologicky rozložitelného podílu komunálního odpadu. Nevýhodou těchto materiálů jsou ovšem jejich horší zpracovatelské a mechanické vlastnosti, vyšší hmotnost např. obalů a vyšší cena [1] [2].

I když se na trhu již objevují biodegradabilní komerční polymery [3], stále je podíl těchto rozložitelných obalových materiálů, které jsou spotřebiteli nabízeny, nedostačující. Nebude-li vyvíjen tlak na firmy, představující hlavní producenty obalových materiálů, aby změnilu svou výrobní politiku, nebude možné dostát závazkům vůči EU dle směrnice Evropského parlamentu a rady (ES) č. 98/2008 o odpadech, která stanovuje opatření na ochranu životního prostředí a lidského zdraví předcházením nepříznivým vlivům vzniku odpadů a nakládání s nimi.

Konkrétní aplikace často užívaných konvenčních plastů (Tab. 1), považovaných převážně za nebiodegradabilní, a biodegradabilních polymerních materiálů (Tab. 2) [4], jsou přehledně shrnuty níže.

**Tab. 1** Přehled každodenně využívaných plastových materiálů, převážně nebiodegradabilních (upraveno dle [4])

Typ polymeru	Užití/aplikace
Polyethylen (PE)	Velmi široká aplikace, jelikož je levný
Nízkomolekulární polyethylen (LDPE)	Venkovní nábytek, podlahová dlažba, obalové a zemědělské fólie, láhve, trubky, desky
Vysokomolekulární polyethylen (HDPE)	Vstříkovací výrobky denní potřeby, velkoobjemové nádoby, pytle, mikrotenové sáčky
Polypropylen (PP)	Brčka na pití, kelímky na jogurty, nárazníky automobilů, trubky, fólie, vlákna
Polystyren (PS)	Obaly, potravinové misky, kelímky, podnosy, plastové nádobí na jedno použití, přístroje, boxy na CD a kazety
Akrylonitril butadien styren (ABS)	Kanalizační potrubí, elektronické přístroje, např. počítačové monitory a klávesnice, tiskárny.
Polyvinylchlorid (PVC)	Kanalizační potrubí, sprchovací závěsy, okenní rámy, podlahoviny
Polymethylmetakrylát (PMMA)	Kontaktní čočky, části svítidel, kabiny letadel, zubní technika, výroba plexiskla
Polytetrafluoretylen (PTFE)	Těsnění, antiadhezní vrstvy (teflon)

**Tab. 1** Přehled každodenně využívaných plastových materiálů, převážně nebiodegradabilních (pokračování, upraveno dle [4])

Typ polymeru	Užití/aplikace
Polyetereterketon (PEEK)	Použití v letecké a vojenské technice, implantáty, jeden z nejdražších komerčních polymerů
Polyethyltereftalát (PET)	Láhve, vlákna, fólie
Polykarbonáty (PC)	CD, ochranné štíty a okna, semaforey, čočky, fólie
PC/ABS	Směs vytváří pevnější polymer – výroba vnitřních a vnějších částí automobilů, výroba těla mobilních telefonů
Polyamidy (PA)	Textilní a jiná vlákna (silony – rybářství), štětiny kartáčků na zuby, žíně
Polyuretany (PUR)	Výroba molitanu, technická vlákna (vlasce, kartáče), izolační pěny, válce v tiskárnách
Fenoplasty (PF)	Brzdové materiály, lepidla, bakelit, elektrické zástrčky, lisovací hmoty
Močovinoformaldehydové pryskyřice (UF)	Lisovací hmoty, ztužovačla textilu a papíru, lékařská pojiva, dřevotřískové desky
Melaminformaldehydové pryskyřice (MF)	Finální povrchy stolů a nábytku, podložky v elektrotechnice, náhrada keramických hrníčků, tácků, misek (pro děti)

**Tab. 2** Aplikace biodegradabilních polymerních materiálů (upraveno dle [5])

Typ polymeru	Užití
Kyselina polyglykolová (PGA)	Vstřebatelné nitě, kontrolovatelné uvolňování léčiv, implantáty
Kyselina polymléčná (PLA)	Mulčovací fólie, kompostovatelné pytle, systém postupného uvolňování pesticidů a hnojiv
Polykaprolakton (PCL)	Mulčovací a jiné zemědělské fólie, nádoby pro sazenice, pro výrobu systému pomalu se uvolňujících léčiv
Polyhydroxybutyrát (PHB)	Láhve, sáčky, obalový materiál, pleny na jedno použití, nosič pro kontrolované uvolňování léčiv
Polyhydroxyvalerát (PHV)	Biochemické aplikace, systém pro kontrolované uvolňování léčiv a insekticidů
Polyvinylalkohol (PVA)	Vlákna, zahušťovačlo a stabilizátor suspenzí, chirurgické nitě, výroba barev a lepidel, apretace papíru a textilu
Polybutylen adipát tereftalát (PBAT)	Kompostovatelné pytle, fólie pro zemědělské účely, pro průmysl rychlého občerstvení

## 1.2. Biodegradace polymerních materiálů

Pod pojmem biodegradace jsou zahrnuty procesy, kterými dochází k odbourávání organických látek působením mikroorganismů (nejčastěji pomocí mikroskopických hub a bakterií) [4]. Biodegradace bývá ovlivněna teplotou, přítomností kyslíku, živin, vlhkostí, pH, světlem, ale také procesy jako je abiotická oxidace, fotooxidace či mechanické narušování materiálu apod. [6]. Polymerní látky, zvláště ty syntetické, představují pro mikroorganismy materiál obecně špatně přístupný a rozložitelný. Problém představuje především velká

molekulová hmotnost, dlouhé řetězce, hydrofobnost polymerů a jejich chemická struktura. Rychlost biodegradace bývá také ovlivněna síťováním a větvením řetězce polymerní molekuly či krystalickým a semikrystalickým uspořádáním polymerů [7].

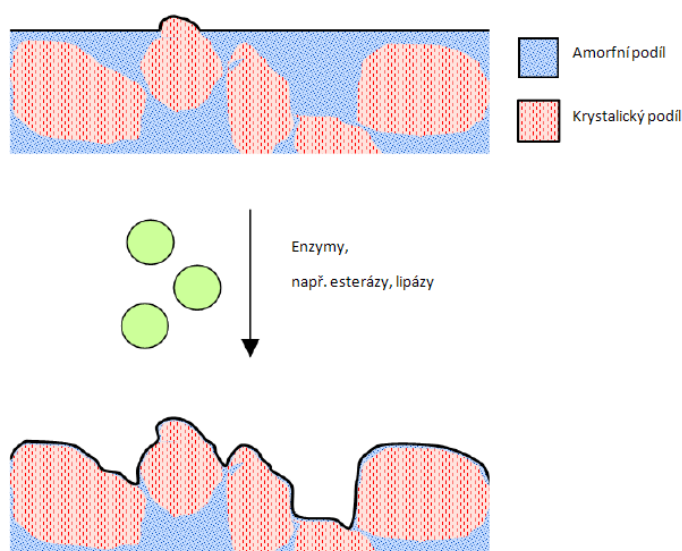
Biodegradace polymerních materiálů probíhá v několika na sebe navazujících krocích:

- **biodeteriorace**- vlivem MO a/nebo abiotických faktorů dochází k dezintegraci materiálu tvorbou trhlin, pórů, atd.
- **depolymerizace**- působením MO a jejich katalytických látek (specifických extracelulárních enzymů) a popřípadě volných radikálů nebo abiotických faktorů (světlo, teplo, chemická činidla) dochází ke štěpení polymerních molekul na oligomery, dimery či monomery, které jsou schopné poté projít přes buněčnou stěnu nebo cytoplasmatickou membránu mikroorganismu a tím být využity v dalším kroku biodegradace
- **asimilace**- začlenění transportovaných molekul do metabolismu mikroorganismu v cytoplazmě, nastává produkce energie, biomasy a tvorba primárních a sekundárních metabolitů
- **mineralizace**- konečné produkty, jako  $\text{CO}_2$ ,  $\text{CH}_4$ ,  $\text{N}_2$ ,  $\text{H}_2\text{O}$  a další soli jsou vylučovány do extracelulárního prostředí [6, 8].

### 1.2.1. Enzymatická biodegradace

Zvláštním druhem biodegradace je biodegradace uskutečňovaná pomocí mikrobiálních enzymů, makromolekul katalyzujících chemické reakce. Mikrobiální enzymy představují biologická agents, které poskytují účinný nástroj pro biodegradaci polymerů, aniž by byly dále ohroženy jiné složky životního prostředí [7, 9].

Všechny mikroorganismy produkují a v různé míře uvolňují, do vnějšího prostředí, enzymy v širokém spektru aktivit. Kromě podmínky strukturální příbuznosti štěpitelných vazeb s vazbami v přírodních substrátech, enzymatická biodegradace polymerů závisí i v tomto případě na přístupnosti jednotlivých molekul polymeru pro interakci s enzymem. Je známo, že se zvyšujícím se stupněm krystalizace polymeru je limitován pohyb řetězců, čímž se snižuje schopnost biodegradčních agens (např. mikrobiálních lipáz) napadat tyto řetězce (Obr. 1) [10, 11].



**Obr. 1** Schematické znázornění vlivu krystalizace polymeru na enzymatickou biodegradaci. Mikrobiální enzymy jsou schopny rozkládat amorfní podíl polymeru a hloubka biodegradace je ovlivněna přítomností krystalického podílu (upraveno dle [10])

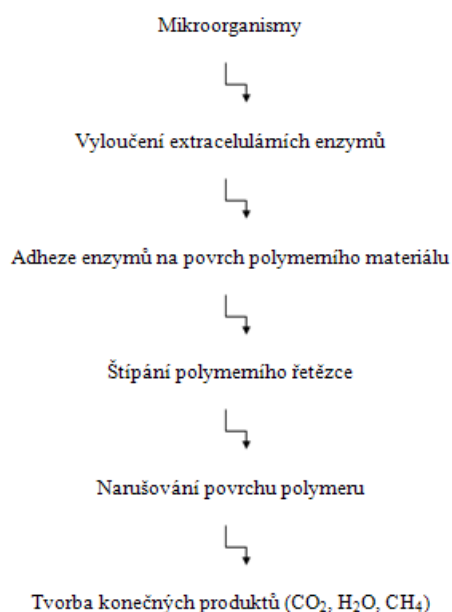
Z pohledu biodegradace polymerních materiálů lze enzymy, které se aktivně podílejí na tvorbě nízkomolekulárních látek, rozdělit do dvou kategorií: extracelulární a intracelulární depolymerázy [12].

Z nich např. enzym laktáza produkovaná aktinomycetami *Rhodococcus ruber* by se mohla uplatnit při biodegradaci PE, kde by byla nápomocna v oxidaci hlavního C-H řetězce [9, 13]. Jako další enzymy, eventuelně nápomocny při biodegradaci PE, jsou uváděny proteázy, produkované druhy *Brevibacillus* sp. a *Bacillus* sp. [13]. Lipáza produkovaná druhem *Rhizopus delemer* a polyuretanová esteráza produkovaná *Comamonas acidovorans* je zodpovědná za degradaci nízkomolekulární PLA, zatímco vysokomolekulární PLA degraduje druh *Amylotopsis* sp. [9, 12]. Další enzymy zodpovědné za degradaci různých typů polymerů jsou uvedeny v Tab. 3.

**Tab. 3** Přehled vybraných druhů hub a bakterií účastnících se biodegradace polymerů (upraveno dle [9])

Zdroj	Enzym	Mikroorganismus	Substrát	Lit.
<b>Houby</b>	glukosidáza	<i>Aspergillus flavus</i>	PCL	[13]
	kutináza	<i>Aspergillus oryzae</i>	Polybutylen jantaran (PBS)	[14]
	kataláza, proteáza	<i>Aspergillus niger</i>	PCL	[13]
	neznámý	<i>Streptomyces</i>	PHB, PCL	[13]
	ureáza	<i>Trichoderma</i> sp.	PUR	[15]
	kutináza	<i>Furasium</i>	PCL	[16]
	neznámý	<i>Amycolaptosis</i> sp.	PLA	[16]
<b>Bakterie</b>	serinová hydroláza	<i>Pestalotiopsis microspora</i>	PUR	[17]
	lipáza	<i>Rhizopus delemer</i>	PCL	[13]
	neznámý	<i>Firmicutes</i>	PHB, PCL, PBS	[13]
	lipáza	<i>Penicillium, Rhizopus arrizus</i>	Polyethylen adipát (PEA), PBS, PCL	[13]
serinová hydroláza	<i>Pseudomonas stutzeri</i>	Polyhydroxy- alkanoáty (PHA)	[16]	

Zjednodušeně lze mechanismus enzymatické biodegradace popsat ve dvou fázích. Mikroorganismy nejprve vyloučí extracelulární enzymy, které se adherují na substrát (polymerní materiál) a poté dojde k hydrolytickému štěpení materiálu (Obr. 2) [7, 9].



**Obr. 2** Zjednodušený mechanismus enzymatické biodegradace polymerů (upraveno dle [9])

### 1.3. Vybrané typy biodegradabilních polymerů

#### 1.3.1. Polyethylen (PE) s prooxidanty

PE je obecně považován za polymer nepodléhající biodegradaci a to nejen proto, že má vysokou molekulovou hmotnost, je hydrofobní a neobsahuje polární funkční skupiny, které by mohly být napadnutelné enzymy mikrobiálního společenstva, ale také právě kvůli obsahu antioxidantů a stabilizátorů, které zabraňují oxidaci PE vlivem účinků vnějšího prostředí [20].

V 30. letech 20. století byly intenzivně zkoumány možnosti stabilizace PE, jelikož podléhal vlivům prostředí a byl nestálý. Tato problematika byla vyřešena přidávkou antioxidantů a stabilizátorů při výrobním procesu. Nicméně, již koncem 80. let 20. století byly syntetické polymery tak hojně využívány, že se jimi bylo nutné zabývat, vzhledem k tomu, že začaly tvořit podstatnou část komunálního odpadu [21].

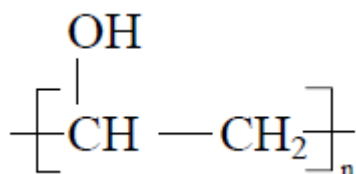
Pokud PE modifikujeme přidávkou prooxidantů, které zvyšují citlivost polymerního řetězce vůči termo- a/nebo foto-oxidaci, snižuje se odolnost polymeru vůči abiotickým vlivům prostředí a dochází ke vzniku biodegradabilních nízkomolekulárních produktů. Tyto produkty jsou již více hydrofilní a mohou přestupovat do vodného prostředí a tím i do buněk mikroorganismů [22], kde nastává jejich úplná mineralizace.

Podstatou prooxidantů jsou ionty přechodových kovů, většinou přidávaných ve formě organických komplexů (např. stearáty  $\text{Fe}^{3+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$ ). Komplexy  $\text{Fe}^{3+}$  či  $\text{Cu}^{2+}$  přidávané do matrice PE podporují foto-oxidaci materiálu, zatímco komplexy  $\text{Mn}^{2+}$  a  $\text{Co}^{2+}$  jsou nezbytné pro zahájení termo-oxidace [23, 24].

Navzdory množství publikovaných prací zabývajících se biodegradací PE, není stále jisté, zda mikroorganismy hrají hlavní roli při jeho rozkladu. Prokázán byl ovšem růst MO na povrchu polymeru a také degradace nízkomolekulárních produktů abiotické oxidace pomocí *Arthrobacter paraffineus* [25] či *Rhodococcus rhodochrous* [26].

Biodegradabilita PE je zkoumána nejčastěji v prostředí půdy či kompostu. V současné době je otázka jejich biodegradability a především časového horizontu těchto přeměn stále otevřená, nicméně se zdá, že tyto materiály nemohou být v souladu se současnými technickými normami definujícími pojem „biodegradabilní polymerní materiál“.

#### 1.3.2. Polyvinylalkohol (PVA)



Obr. 3 Vzorec PVA

Polyvinylalkohol (Obr. 3) je syntetický polymer rozpustný ve vodě. PVA není získáván přímo polymerací monomeru. Polymer vzniká hydrolýzou roztoku polyvinylacetátu v kyselém nebo zásaditém prostředí [27]. Díky hydrofilnosti polymerního řetězce je PVA široce využíván v průmyslových aplikacích jako adhezivum, v textilním a papírenském průmyslu, zlepšuje povrchy filmů a používá se k výrobě barev a lepidel na vodní bázi. Svým chováním v mnohém připomíná želatinu, proto se používá např. v potravinářském průmyslu pro výrobu ovocného želé, v chemickém průmyslu jako ochranný koloid pro suspenzní polymerace a jelikož je to polymer nerozpustný v olejích, tucích, nebo benzínu používá se pro přípravu impregnačních hmot [28].

Biodegradace PVA se začala zkoumat již před 70. lety. Prvním mikroorganismem, který byl v této souvislosti identifikován byla houba *Fusarium lini* B [29]. Počátkem 70. let 20. století došlo k zintenzivnění výzkumů, které odhalily konkrétní druhy podílející se na rozkladu tohoto syntetického a ve vodě rozpustného polymeru. Mikroorganismy byly izolovány jak z půdy, např. *Pseudomonas borealis* O-3 [30, 31], tak z říční vody (*Alcaligenes fecalis* KK314 [30]). Dalším významným rodem podílejícím se na utilizaci PVA je rod *Sphingomonas* [33].

Polyvinylalkohol není toxický, jeho zvýšená koncentrace v odpadních vodách je ovšem nežádoucí. PVA nejspíš prochází procesem čištění na čistírně odpadních vod, aniž by byl zcela odbourán. Jeho zvýšené množství ve vodě vede k produkci pěny, která brání přestupu kyslíku do vody a představuje vážnou hrozbu pro vodní organismy [34].

Výzkum biodegradace PVA pokračuje i v dnešní době, což lze dát do souvislosti s rostoucí spotřebou tohoto polymeru zvláště v Asii, západní Evropě a USA [34]. Odbourávání PVA z životního prostředí nebrání jeho chemická struktura, jako tomu je u většiny polymerů. Nedostačující jsou informace o možné biodegradaci za podmínek anaerobních [35].

### 1.3.3. Polyestery (PES) a kopolyestery

Polyestery představují skupinu plastů s širokým využitím. Z pohledu komerčního využití jsou především používány aromatické polyestery, jako poly(etylen tereftalát) (PET) nebo poly(butylem tereftalát) (PBT), pro jejich vynikající mechanické vlastnosti. Tyto polymery jsou ale doposud považovány za nebiodegradabilní [36].

Změnu představuje kombinace alifatických a aromatických monomerů, kdy vzniká kopolyester vyznačující se výbornými mechanickými vlastnostmi s dobrou biodegradabilitou [37] např. poly(butylem adipát tereftalát) (PBAT) či podobný materiál s komerčním názvem Ecoflex [38].

Významným zástupcem polyesterů, je kyselina polymléčná (PLA). PLA a její kopolymery jsou v posledních desetiletích ostře sledovány a to zejména proto, že je možné ji vyrobit z obnovitelných zdrojů (cukrová řepa, pšenice [39]), její mechanické vlastnosti jsou podobné polyolefinům a má schopnost se rozložit jak v lidském těle, tak v přírodě [40].

Biodegradabilním plastem vyráběným z obnovitelných zdrojů je také poly( $\beta$ -hydroxybutyrát) (PHB). Jedná se o termoplastický polyester, netoxický a ve vodě nerozpustný polymer. Jako biokompatibilní polymer se široce využívá ve zdravotnictví (implantáty, chirurgické nitě) a ve farmacii. Svými zpracovatelskými a užitnými vlastnostmi se podobá polypropylenu (PP), avšak jeho rychlá termická degradace v blízkosti teploty tání, fyzikální stárnutí a vysoká cena brání jeho většímu uplatnění, např. v obalové technice [41]. Lepších mechanických vlastností PHB, lze dosáhnout přípravou směsí tohoto polymeru s jiným materiálem, např. s polyhydroxyvalerátem (PHV) [42] či  $\epsilon$ -kaprolaktonem [41].

Zástupcem alifatických polyesterů vyráběných z petrochemických prekurzorů je polykaprolakton (PCL). I když tento plast není vyráběn z obnovitelných zdrojů, je považován za zcela biodegradabilní, především v prostředí aktivovaného kalu, kompostu, půdy a mořské vody [43]. Jelikož má PCL nízkou teplotu tání ( $T_t = 59-64^\circ\text{C}$ ) jeho využití, jako náhrady za konvenčně využívané nekompostovatelné plasty je obtížné. Využívá se spíše v kombinaci s jinými polymery, např. se škrobem. Tímto je možné dosáhnout lepších zpracovatelských a mechanických vlastností [44].



## 2. METODY STUDIA BIODEGRADACE POLYMERŮ

Posuzovat biodegradabilitu materiálů lze pomocí metod založených na studiu rozličných charakteristik, například na redukci průměrné molekulové hmotnosti [45], změně mechanických vlastností a povrchu materiálu [46], produkci CO<sub>2</sub> [47], zkoumání mikrobiálního ataku [48, 49], apod. Pro zkoumání biodegradace materiálu lze také použít metody molekulární biologie, které jsou rychlé, spolehlivé, reprodukovatelné a relativně levné [50].

Některé postupy založené na výše uvedených metodikách jsou normovány světovými standardizačními institucemi, např. ISO (International Organization for Standardization), ASTM (American Standard Test Method), CEN (European Standardization Committee) [51].

### 2.1. Metody založené na materiálové charakterizaci polymerů

Primární vliv na rozklad polymerů v procesu biodegradace má jejich struktura. Určení podílu krystalické a amorfni složky polymerního materiálu může sloužit k bližšímu odhadu chování materiálu v procesu biodegradace. Biodegradaci nejnáze podléhá amorfni podíl materiálu, jelikož části řetězců v těchto místech mají větší možnost pohybu a také jsou volněji uspořádány. Takto je umožněna rychlejší difuze malých molekul, např. vody, do materiálu. K určení stupně krystalinity lze využít termoanalytickou metodu, diferenciální skenovací kalorimetrii (DSC) či rentgenovou difrakci [52].

Míru biodegradace je možné pozorovat změnou mechanických vlastností materiálu. Určujícím faktorem může být snížení molekulové hmotnosti, což vede např. ke snížení pevnosti materiálu v tahu [53].

Biodegradaci materiálu lze posuzovat také užitím gelové permeační chromatografie (GPC). GPC umožňuje určit distribuční křivku molekulové hmotnosti polymeru a z jejich změn v průběhu procesu lze hodnotit charakter biodegradace, např. tvorbu nízkomolekulárních frakcí [53].

K získání vizuálních a kvantitativních charakteristik o struktuře povrchu materiálu bývá využíván optický a skenovací elektronový mikroskop (SEM). SEM poskytuje detailnější informace o této struktuře, např. charakterizuje topografii povrchu (přítomnost trhlin, děr), krystalickou strukturu, chemické složení atd. Pomocí SEM je možné sledovat tvorbu biofilmu na studovaných polymerech [54].

Další chemické změny, které mohou nastat v materiálu během biodegradace, lze studovat infračervenou spektroskopií (IČ) [55]. Pomocí IČ lze sledovat vznik nových chemických vazeb či skupin. Např. v případě PE lze nárůstem karbonylového indexu, který je získán výpočtem z podílu absorbcí karbonylového píku (vlnčet 1713 cm<sup>-1</sup>) a píku -CH<sub>2</sub>- (vlnčet 1465 cm<sup>-1</sup>) určit míru oxidace testovaného polymeru během biodegradčního procesu. Z grafické závislosti karbonylového indexu na čase, po který byl vzorek vystaven abiotické oxidaci lze pozorovat, jak se např. s dobou termooxidace zvyšuje stupeň zoxidování materiálu [23].

### 2.2. Metody založené na produkci plynů

Tyto metody mají nezastupitelný význam v posuzování biodegradability polymerů. Lze jimi studovat konečnou mineralizaci materiálu na základě vyprodukovaného množství CO<sub>2</sub> či spotřebovaného O<sub>2</sub>. Podíl mineralizace odpovídá vyvinutému množství oxidu uhličitého z posuzovaného materiálu. Ze zjištěného množství vyprodukovaného CO<sub>2</sub> je přepočítána mineralizace uhlíku z materiálu, na jeho základě teoretického množství v materiálu, který může být určen elementární analýzou nebo výpočtem [56].

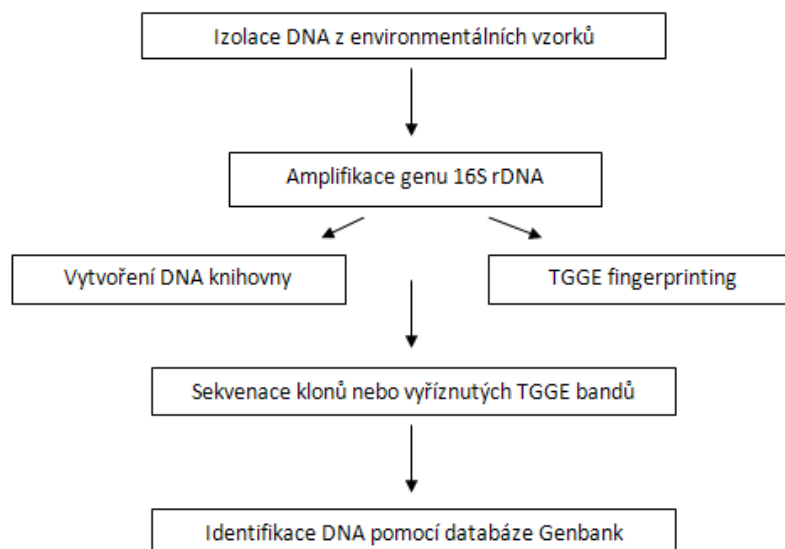
Vyprodukovaný CO<sub>2</sub> může být měřen respirometrickou metodou kumulativní (CMP- *cumulative measurement respirometric*) či přímou (DMP- *direct measurement respirometric*). V případě metody CMP je vyvinutý CO<sub>2</sub> zachycován do roztoku NaOH či Ba(OH)<sub>2</sub> a poté je rozpuštěný CO<sub>2</sub> titrován známou koncentrací H<sub>2</sub>SO<sub>4</sub>. Metoda DMP využívá k určení produkce CO<sub>2</sub> plynovou chromatografii (GC) či IČ spektroskopii [56, 57].

### 2.3. Možnosti použití molekulárně biologických metod

Metody molekulární biologie nachází využití zejména k identifikaci a monitorování mikroorganismů při biodegradaci v přirozených prostředích, například půdě. K identifikaci se nejčastěji používá amplifikace genu 16S rDNA, protože tento gen je přítomen ve všech bakteriích a existují velmi bohaté databáze jeho sekvencí.

PCR amplifikace genu 16S rDNA umožňuje pochopit složení společenstev, především mikroorganismů přítomných v prostředí, které nejsou kultivovatelné a není je tedy možno studovat metodami klasické mikrobiologie [58].

Mezi nejvíce používané kultivačně nezávislé metody patří DGGE (gelová elektroforéza v gradientu denaturačních činidel) či TGGE (gelová elektroforéza v teplotním gradientu). Výhodou TGGE je, že metoda pracuje na jednodušším principu a je vhodná pro separaci dlouhých řetězců DNA a RNA [58, 59]. Před použitím metody DGGE či TGGE je nutná izolace DNA a PCR amplifikace fragmentu vhodného genu. Jednou z předností těchto technik je možnost izolace DNA fragmentů z jednotlivých bandů a jejich následná identifikace (Obr. 4).



**Obr. 4** Schéma na sebe navazujících kroků experimentu od izolace DNA z environmentálního vzorku, po konečnou identifikaci fragmentu DNA z TGGE gelu

Jako další molekulárně biologickou metodu, která se používá pro výzkum biodegradací, je možné zmínit RFLP (restriction fragment length polymorphism). Technikou lze obdobně, jako u TGGE či DGGE, získat fingerprint komplexního mikrobiálního společenstva [60].

#### 2.3.1. Izolace DNA

Prvním krokem většiny molekulárně biologických metod je izolace genetického materiálu. Izolace DNA z environmentálních vzorků spočívá v extrakci nukleových kyselin z buněk mikroorganismů, odstranění kontaminujících složek a eliminace sloučenin, které

inhibují či snižují citlivost následujících analýz (polymerázová řetězová reakce, hybridizační techniky, apod.) [61].

Jelikož je k dispozici nepřeberné množství izolačních protokolů, které mohou významně ovlivnit složení mikrobiální komunity, volba vhodné techniky je klíčovou záležitostí [62]. Výběr závisí na povaze zkoumaného vzorku [63, 64, 65] a na účelu, ke kterému budeme dále s DNA pracovat. Např. užití ultrazvuku [66] či techniky kombinující mletí, zmrazování a tání [62] vzorku pro rozrušení buněk MO se jeví být úspěšné v případě, kdy pracujeme s půdou s vyšším obsahem jílu/bláta a chceme-li získat DNA o vysoké molekulové hmotnosti (cca 20 kb) [61, 62, 63, 67]. Zvolíme-li k fyzikálnímu rozrušení buněk kuličky (nejčastěji skleněné), získáme obecně vyšší výtěžek nukleové kyseliny, která je tvořena převážně krátkými fragmenty DNA [68]. Nicméně, tento způsob lyze je účinnější pro  $G^+$  bakterie, jelikož dokáže rozrušit jejich komplikovanější buněčnou stěnu, tvořenou silnou vrstvou peptidoglykanu.

Pro extrakci DNA je možno využít různé techniky, např. lze zmínit fenol-chloroformovou extrakci a použití chromatografických kolonek. Pomocí fenol-chloroformové metody se extrahují nukleové kyseliny rozpuštěné ve vodném prostředí a odstraňují se ostatní složky lyzátu, např. proteiny, lipidy, nízkomolekulární látky [61]. Extrakce DNA pomocí kombinace chromatografických kolonek a centrifugace bývá využíváno v komerčně dostupných kitech.

Vzrůstající počet publikací zabývajících se izolačními protokoly a extrakčními technikami, ukazuje na důležitost těchto kroků v molekulární biologii a to především z toho důvodu, že volbou extrakčního protokolu je možné ovlivnit výslednou fylogenetickou diverzitu získané DNA [62, 69, 70]. Tudíž, čím více „drastický“ izolační protokol použijeme, tím je větší pravděpodobnost získání DNA, např. z  $G^+$  bakterií, ze zkoumaného vzorku.

### 2.3.2. TGGE

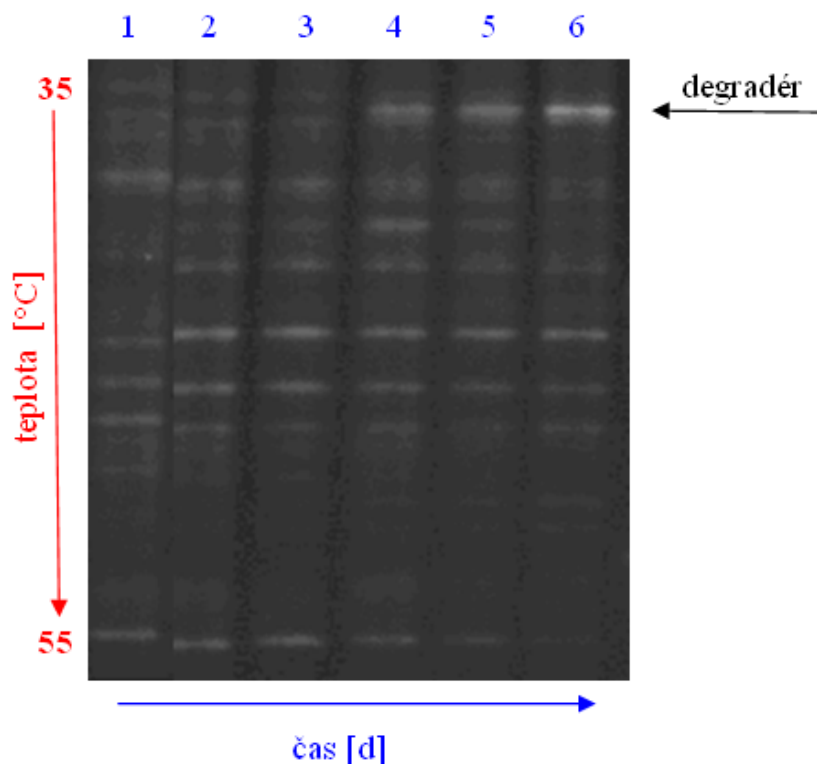
TGGE představuje techniku používanou v molekulární biologii. Lze pomocí ní rychle a spolehlivě charakterizovat složení mikrobiálního společenstva a studovat jeho změny v prostoru a čase [71].

Metoda je založena na separaci DNA fragmentů o stejné, či podobné velikosti, které se liší pořadím nukleotidů. Směs DNA fragmentů se dělí elektroforeticky v akrylamidovém gelu, který obsahuje denaturační činidla: močovinu a formamid. V závislosti na obsahu denaturačních činidel v gelu, teplotě a obsahu G-C a A-T párů v ampliconech, dochází k jejich postupné denaturaci a jejich zastavení v gelu. Z původní dvouřetězcové molekuly se stává částečně jednořetězcová struktura.

Aby byly zlepšeny detekční schopnosti TGGE, obsahuje jeden z primerů, tzv. forward, GC-svorku, tvořenou GC nukleotidy [72, 73]. Ta je připojena k 5'-konci primeru a má délku asi 40 párů bází (bp) [70]. Při postupu DNA fragmentů akrylamidovým gelem nenastane v oblasti GC-svorky úplné rozvolnění dvouřetězcové struktury DNA (dsDNA) na jednořetězcovou (ssDNA). Dojde pouze ke skokovému snížení mobility tohoto DNA fragmentu v gelu. Pomocí GC svorky se signály jednotlivých bandů stávají ostřejšími [74].

Na TGGE systému lze vzorky separovat buď paralelně či kolmo vůči teplotnímu gradientu. Zatímco kolmá TGGE je hlavně užívána k optimalizaci podmínek separace, paralelní TGGE (Obrázek 4) poskytuje rychlou analýzu různých vzorků [75].

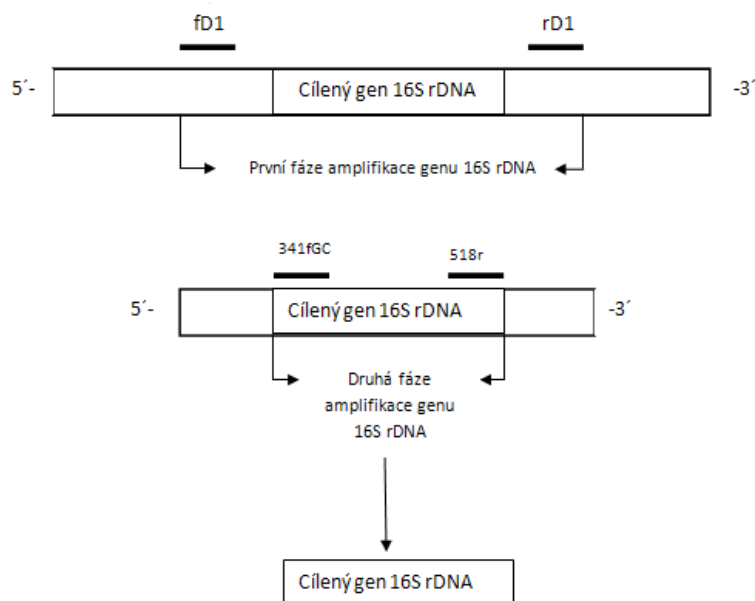
Výsledkem separace je tzv. fingerprint, který je složen z jednotlivých bandů, které reprezentují dané mikrobiální společenstvo [70, 71], (Obr. 5).



**Obr. 5** Výsledný paralelní TGGE gel z procesu biodegradace polymerního materiálu. Dráhy 1-6 představují vzorky odebrané v daných dnech biodegradace. Jednotlivé bandy zobrazují signály dominantních druhů mikroorganismů během procesu. V horní části gelu je patrné zesílení intenzity bandy, což poukazuje na množení určitého MO, který využívá polymer pro svůj růst

Fragmenty DNA nanášené na TGGE gely jsou obvykle produkty PCR. V závislosti na původu amplifikovaného DNA fragmentu vzorku a očekávané velikosti ampliconů jsou voleny příslušné primery. Je možné použít primery univerzální či specifické. S pomocí univerzálních primerů [72] můžeme například amplifikovat 16S rDNA gen všech eubakterií ve vzorku. Specifické primery použijeme k získání informací o zvolených taxonomických skupinách mikroorganismů. Tzn. ve vzorcích z biodegradace PLA budeme očekávat důležitost aktivity aktinomycet, jako dominantních degradérů. Pro amplifikaci cílených fragmentů DNA tedy použijeme specifické primery pro aktinomycety [76], abychom na TGGE gelu získali signály odpovídající této skupině mikroorganismů.

Vyšší specifčnosti a zvýšení množství připravených PCR produktů nanášených na TGGE gely, je možno dosáhnout modifikací techniky polymerázové řetězové reakce, tzv. nested PCR (Obr. 6). Amplifikace genu 16S rDNA probíhá v tomto případě dvoufázově. V první fázi, s 20-30 amplifikačními cykly, je pomocí jedné dvojice primerů namnožena delší sekvence nukleové kyseliny. Takto získané amplicony jsou přeneseny do nové amplifikační zkumavky obsahující druhou dvojici primerů, specifických k vnitřnímu úseku ampliconů. Ve druhé fázi, opět s 20-30 amplifikačními cykly, získáme amplicony s kratší vnitřní sekvencí. Touto modifikací PCR lze dodatečně zředit inhibitory polymerázy. Nevýhodou nested PCR může být vyšší riziko kontaminace [77].



**Obr. 6** Princip nested PCR

Jedním z dalších faktorů, který ovlivňuje výsledný TGGE gel je systém použitých pufrů. Zvolit lze např. TAE (tris- acetát – EDTA) či TBA (tris- borát- EDTA) pufr.

Vizualizaci DNA fragmentů v TGGE gelu lze provádět různými barvicími technikami. Je možné použít interkalační barviva jako např. ethidiumbromid, SYBR Green, GelRed nebo barvit gely pomocí stříbra. Jednotlivé techniky se od sebe liší citlivostí. Za velmi citlivou metodu je považována detekce fragmentů DNA pomocí stříbra, které obarví i ty nejméně výrazné fragmenty DNA. Nevýhodou tohoto barvení je jeho časová náročnost, finanční stránka a skutečnost, že takto obarvené gely lze obtížně použít pro další práci s DNA [78]. Alternativou, umožňující sledovat méně dominantní fragmenty DNA, může být barvivo GelRed. Barvivo nezpůsobuje obarvení pozadí a lze jej bezpečně likvidovat [78].

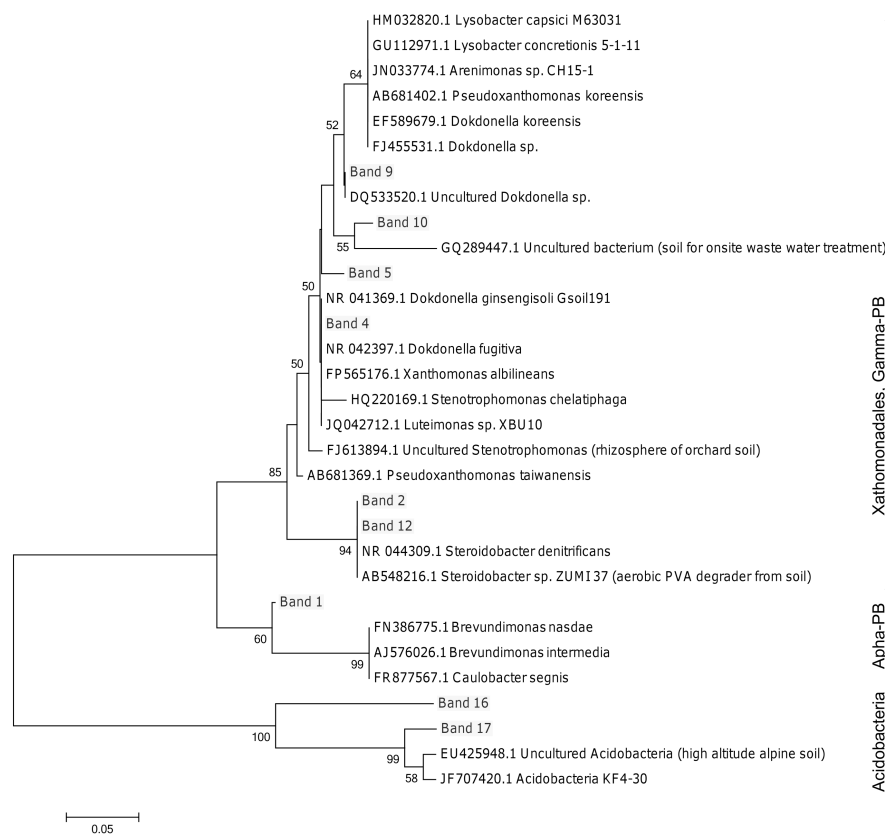
Silnou stránkou techniky TGGE je možnost izolace DNA z jednotlivých bandů. Místo v gelu obsahující zajímavý band může být vyříznuto a DNA z tohoto kousku gelu izolována a po přečištění a sekvenaci, identifikována. Tento postup má však svá úskalí, v podobě možné přítomnosti většího množství sekvencí obsažených v jednom bandu, časové náročnosti a degradace DNA vlivem UV záření [72, 75].

Metoda je dále využívána i v jiných výzkumných oblastech jako je mikrobiální ekologie, molekulární biologie, lékařství (sledování mutací) [72], apod.

### 2.3.3. Fylogenetická analýza

Studium vzájemných vztahů mezi různými taxonomickými jednotkami lze uskutečnit pomocí fylogenetické analýzy. Příbuzenské vztahy lze posuzovat např. na základě genetické příbuznosti [79].

Grafickým znázorněním fylogenetické analýzy je fylogenetický strom (Obr. 7).



**Obr. 7** Fylogenetický strom sekvencí získaných z TGGE analýzy

V některých fylogenetických stromech mohou místo taxonomických jednotek vystupovat přímo jednotlivé biologické druhy nebo i jednotlivé geny [79].

V našem případě je ve fylogenetickém stromu vizualizována příbuznost mezi sekvencemi bází genu kódujícího 16S rDNA nalezenými ve vzorku.

#### 2.3.4. Tvorba a sekvenace DNA knihoven

Vložíme-li specifický fragment DNA do klonovacího vektoru (plazmidu, kosmidu, bakteriofágu, apod.) a tuto rekombinantní molekulu DNA pomnožíme v hostitelské buňce (nejčastěji bakterii) získáme identické kopie neboli klony. Soubor těchto rekombinantních plasmidů nesoucí vložené fragmenty DNA představuje tzv. DNA knihovnu [80].

Tvorbou takovýchto DNA knihoven lze alternativně zkoumat společenstvo mikroorganismů při biodegradacích. Směs DNA fragmentů se rozdělí do klonů, ty se pomnoží a pak se sekvenují. Nejčastěji se tímto způsobem zkoumají právě sekvence genu 16S rDNA.

#### 2.3.5. Real-time PCR (RT-PCR)

Stanovení množství určité sekvence DNA ve vzorku, se provádí pomocí tzv. real-time PCR (též označována jako kvantitativní PCR; qPCR). Množství ampilifovaného produktu je sledováno pomocí fluorescenčních sond či barviv na základě změny intenzity fluorescenčního záření, která je přímo úměrná množství produktu. Prakticky se tento druh PCR provádí na cyclerech, kdy se v každém cyklu PCR detekuje fluorescence. Kvantifikace se provádí pomocí amplifikačních křivek, které vznikají vnesením fluorescence oproti pořadí cyklu [81].

Tato metoda znamenala převrat v molekulární diagnostice, právě kvůli možnosti kvantifikovat množství určité sekvence DNA ve vzorku [81].

### 3. CÍLE PRÁCE

Cílem předkládané disertační práce bylo studium biodegradovatelných polymerních materiálů, především jejich biodegradace v prostředí půdy, kompostu případně ve vodném prostředí. Zavést a aplikovat vybrané molekulárně biologické metody pro studium mikrobiologie biodegradace polymerů. Našimi cíly bylo:

1. Sledovat biodegradaci připravených abioticky oxidovaných vzorků polyethylenů s prooxidanty.
2. Zavést vybrané molekulárně biologické metody v laboratoři fakulty a demonstrovat jejich použitelnost pro studium biodegradace polymerních látek. Klíčové zde bylo zavedení metody gelové elektroforesy v teplotním gradientu.
3. Sledovat biodegradaci PVA za denitrifikačních podmínek, případně aplikovat zvolené molekulárně biologické metody pro studium této problematiky.
4. Sledovat mikrobiologii biodegradace PLA v závislosti na jeho formě a distribuci molekulových hmotností pomocí zvolených molekulárně biologických metod.

## 4. ZVOLENÉ METODY ZPRACOVÁNÍ

Studium biodegradace materiálů bylo provedeno pomocí inkubačních testů s plynově chromatografickou koncovkou. Sledován byl úbytek polymeru v čase.

Současně byly aplikovány molekulárně biologické metody pro získání informací o mikrobiologii degradace zkoumaných polymerů. Pomocí metody izolace nukleových kyselin byla získána DNA vzorků a vhodnou PCR metodou byl amplifikován gen 16S rDNA. Technikou TGGE došlo k získání signálů dominantních mikroorganismů účastnících se procesu biodegradace. Paralelně bylo možné výsledky TGGE potvrdit vytvořením knihovny klonů zvoleného genu a sekvenováním zvoleného počtu těchto klonů.

### 4.1. Stanovení míry biodegradace

#### 4.1.1. Úprava vzorku pro biodegradační testy

Abiotická oxidace, která byla provedena v případě studia biodegradace PE, simulovala stárnutí materiálu, za podmínek vystavení vlivům prostředí (světla, tepla), po určitou dobu. Docílena byla redukce molekulové hmotnosti polymeru a tvorba nízkomolekulárních látek, které pak mohly být asimilovány mikroorganismy [54].

#### 4.1.2. Biodegradační testy

Vzorek polymeru byl smíchán s perlitem, který zajistil provzdušňování půdy či kompostu, minerálním médiem, které sloužilo pro dosažení dostatečné vlhkosti prostředí a jako zdroj esenciálních prvků pro mikroorganismy a inokulem.

Biodegradační testy probíhaly v biometrických lahvích (Obr. 8). Po dobu několika měsíců byly z lahví odebírány vzorky plynné fáze, která byla analyzována metodou plynové chromatografie. Zjišťováno bylo množství vyvinutého CO<sub>2</sub> a spotřebovaného O<sub>2</sub>. Množství vyprodukovaného CO<sub>2</sub> v lahvi nám posloužilo pro stanovení stupně biodegradace zkoumaného polymeru.



**Obr. 8** Biometrická láhev pro sledování míry biodegradace polymerů, která je opatřena uzávěrem se septem pro odběr vzorku plynné fáze a dvěma dalšími otvory pro provzdušňování



## 4.2. Stanovení míry biodegradace PVA ve vodném prostředí

Biodegradační testy s polyvinylalkoholem byly prováděny ve vodném prostředí za denitrifikačních podmínek. Biometrické láhve obsahovaly minerální médium, zkoumaný polymer,  $\text{KNO}_3$  jako terminální akceptor pro denitrifikaci a aktivovaný kal, jako zdroj MO. Koncentrace PVA byla v průběhu biodegradace zjišťována jodometricky.

## 4.3. Molekulárně biologické metody

Prvním krokem, pro práci s jednotlivými vzorky, byla extrakce DNA pomocí vhodné metody. Následně byly vzorky amplifikovány metodou PCR, kontrola úspěšnosti postupu byla provedena pomocí agarózové elektroforézy. Získané PCR fragmenty byly separovány s užitím gelové elektroforézy v teplotním gradientu. Po skončení TGGE byly z výsledného gelu vyříznuty bandy, DNA v těchto signálech byla sekvenována a identifikována v databázi GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) pomocí aplikace BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

### 4.3.1. Izolace nukleových kyselin

Klíčovou roli zde hrálo rozrušení membrán mikroorganismů a následná separace a purifikace nukleových kyselin. Byl zvolen kit, který zahrnuje v izolačním protokolu třepání se skleněnými kuličkami (tzv. bead beating), jelikož bylo předpokládáno, že významnou úlohu v biodegradačním procesu hrají  $G^+$  bakterie, u nichž je známo, že mají odolnou buněčnou stěnu. Užitím jiného druhu izolačního protokolu došlo ke zkreslení výsledku.

### 4.3.2. PCR

Získaná DNA byla amplifikována pomocí techniky PCR. Pro vzorky z různých prostředí byla metoda vždy modifikována, tj. byl zvolen vhodný druh PCR metody, vybrány vhodné primery s ohledem na očekávaný výsledek, upravena nasedací, tzv. annealingová, teplota primerů, počet cyklů, apod.

Kontrola úspěšnosti postupu byla uskutečňována pomocí agarózové elektroforézy. Dokumentace výsledků byla provedena pomocí dokumentačního systému.

### 4.3.3. TGGE

PCR fragmenty DNA byly separovány pomocí TGGE Maxi Systému (Obr. 9). Dle povahy separovaných fragmentů byl vytvořen akrylamidový gel o příslušných koncentracích denaturačních činidel. Byl zvolen vhodný rozsah denaturačních teplot. Fragmenty odpovídající přítomným bakteriím byly separovány nejčastěji v rozsahu  $35\text{--}55^\circ\text{C}$  [74], kdežto fragmenty přítomných aktinomycet potřebovaly vyšší teplotní rozpětí  $40\text{--}60^\circ\text{C}$  [76], jelikož obsahují větší podíl GC párů. Dále byla na přístroji nastavena délka separace a elektrické napětí.

Po skončení elektroforézy byl gel neprodleně nabarven vhodnou metodou, např. interkalačními barvivy. K vizualizaci jednotlivých signálů DNA byl použit stejný dokumentační systém, jako při práci s agarózovým gelem.



**Obr. 9** TGGE Maxi Systém pro separaci PCR amplikonů

#### **4.3.4. Excise a purifikace bandů**

Signál na TGGE gelu byl identifikován tak, že konkrétní band byl vyříznut a DNA, obsažená v bandu, byla eluována do roztoku. Způsob eluce DNA byl postupně optimalizován pro jednotlivé vzorky. Eluovaná DNA byla reamplifikována se stejnými primery (s vynecháním GC svorky) a následně byla přečištěna a sekvenována.

#### **4.3.5. Klonování a tvorba DNA knihovny**

Složení mikrobiálního společenstva podléjícího se na zkoumaném procesu lze sledovat také přístupem s vytvořením DNA knihovny.

Metodou PCR byl fragment 16S rRNA genu amplifikován pomocí vhodných primerů ze všech přítomných bakterií. Fragment DNA byl přečištěn pomocí elektroforézy v agarózovém gelu. Restrikční štěpení vektoru (plasmidu, kosmidu, bakteriofágu) a insertu proběhlo jak se získáním lepivých tak tupých konců, dle výběru restrikčních endonukleáz. Připravené fragmenty DNA (tedy izolované vektory a geny) byly spojeny ligací, do funkčních rekombinantních replikonů. Následným procesem transformace byla rekombinantní molekula DNA vložena do živé buňky (bakterie *E. coli*). Zde došlo k několikanásobné replikaci vloženého genu. Následovala selekce transformantů na Petriho miskách s antibiotikem, ke kterému poskytoval gen na klonovacím vektoru resistenci. Pomocí optimalizovaného postupu byla vyizolována plasmidová DNA z vybraných klonů. Pro potvrzení přítomnosti jak vektoru, tak plasmidu ve vzorku (klonu), byla provedena restrikční analýza. Na závěr proběhlo sekvenování zvoleného počtu náhodně zvolených klonů, čímž byla umožněna identifikace většinových mikroorganismů ve zkoumaném společenstvu.

#### **4.3.6. Real-time PCR (RT-PCR)**

Touto metodou bylo stanoveno množství určité sekvence DNA ve vzorku pomocí fluorescenčních sond (interkalačních barviv). Reakce byly složeny ze stejných reagensů jako u klasické metody PCR s tím rozdílem, že byla přidána fluorescenční značka, kterou byla zajištěna možnost kvantifikace vzorku. Analýza byla prováděna na přírodovědecké fakultě Univerzity Karlovy v Praze.

## 5. PUBLIKOVANÉ PRÁCE

Tato kapitola se zabývá stručným popisem čtyř publikovaných prací v odborných časopisech, které vznikly během doktorského studia. Práce jsou rozděleny do dvou tématických celků, vzhledem ke kombinaci použitých metod a materiálů.

První celek, který se skládá ze dvou publikací, je věnován studiu biodegradability polyethylenových fólií obsahující prooxidanty. Biodegradabilita PE byla sledována v prostředí půdy a kompostu pomocí konvenčních metod.

V druhém tématickém celku, obsahujícím rovněž dvě publikace, byl průběh biodegradace sledován s kombinací konvenčních a vybraných molekulárně biologických metod. Konkrétně byla sledována schopnost biodegradace PVA za denitrifikačních podmínek v prostředí aktivovaného kalu a mikrobiologie biodegradace PLA v závislosti na její formě a distribuci molekulových hmotností.

Každá podkapitola je věnována konkrétní publikaci.

### 5.1. Srovnání biodegradability různých PE fólií obsahující prooxidanty (Příloha P1)

Studiu biodegradability byl podroben vysokohustotní (HDPE), nízkohustotní (LDPE) a lineární (LLDPE) polyethylen ve formě tenkých fólií s různým složením a množstvím prooxidantů. Podstatu prooxidantů tvořil organický komplex přechodových kovů v kombinaci  $Mn^{2+}$  a  $Fe^{3+}$ ,  $Mn^{2+}$ ,  $Fe^{3+}$  a  $Co^{2+}$  v různých koncentracích.

V první části studia rozsahu biodegradace PE fólií s prooxidanty byla provedena abiotická úprava jednotlivých vzorků. Polymery byly podrobeny tzv. urychlenému stárnutí ve speciální tomu určené komoře. Dávka záření a stupeň oxidace materiálu odpovídal vystavení venkovnímu prostředí po dobu asi 3 let za podmínek odpovídajícím středoevropskému klimatu v letním období. Působením světla a tepla, tj. fotooxidací a termooxidací, docházelo ke stárnutí polymeru, což mělo za následek křehnutí materiálu vlivem redukce molekulové hmotnosti polymeru a tvorbu nízkomolekulárních produktů. Stupeň oxidace materiálu byl sledován pomocí FTIR, NMR a gelové permeační chromatografie.

Druhá část experimentů byla věnována sledování vlastní biodegradaci PE fólií. Abioticky předupravené PE fólie byly současně podrobeny, jak inkubaci v prostředí minerálního média za účasti čistého kmene bakterie *Rhodococcus rhodochrous*, tak byla sledována jejich schopnost biodegradace v prostředí půdy a kompostu.

Metabolická aktivita bakteriální kultury v minerálním médiu byla zjišťována pomocí stanovení množství adenosintrifosfátu (ATP). Bylo zjištěno, že povaha prooxidantů je hlavním faktorem ovlivňující biodegradaci PE fólií. Určující je zejména vzájemné množství kovů v organickém komplexu. Konkrétně bylo zjištěno, že vyšší koncentrace  $Co^{2+}$  iontů v matici PE fólií vedla k inhibici metabolismu *R. rhodochrous*, tedy nízkým hodnotám ATP a k nízké životaschopnosti buněk na konci experimentu.

K získání informací o průběhu biodegradace PE fólií v komplexním prostředí byly provedeny pokusy v půdě a kompostu. U vybraných polymerů byla biodegradabilita stanovena měřením produkce  $CO_2$ . Paralelně byly testovány referenční látky, které, jak se dalo očekávat, byly ze 100% biodegradabilní, v případě celulózy v horizontu 140 dnů a u hexadekanu 250 dnů v prostředí kompostu. Látkou nejsnáze podléhající biodegradaci byl LLDPE, který byl mikrobiálním konsorciem metabolizován z 24% za dobu 317 dnů. HDPE představoval matici velmi špatně podléhající biodegradaci. Obdobných zjištění bylo dosaženo v biodegradačních pokusech provedených se stejnými polymery v prostředí půdy.

Výsledky získané z obou způsobů inkubace PE fólií byly nejen souhlasné navzájem, ale také se shodovaly se závěry dosaženými pomocí techniky SEM. Byla potvrzena shoda mezi schopností polymeru podléhat biodegradaci a tvorbou biofilmu na povrchu polymeru.

Studie byla uskutečněna ve spolupráci s kolegy na univerzitě v Clermont-Ferrand, předkladatelka disertační práce měla na starosti biodegradační pokusy v komplexních prostředích tj. půdě a kompostu, podílela se rovněž na přípravě a redakci publikace.

## **5.2. Aerobní biodegradace polyethylenových fólií obsahující prooxidanty a uhličitan vápenatý jako plnivo (Příloha P2)**

Hlavním cílem této studie bylo zkoumání vlivu plniva tj. mletého uhličitanu vápenatého v PE na průběh biodegradačních procesů.

Biodegradaci byly podrobeny vzorky LDPE ve formě tenkých fólií s prooxidanty ( $Mn^{2+}$ ) a jeden vzorek obsahoval jemně namletý uhličitan vápenatý.

Fólie byly nejprve předupraveny abiotickou oxidací, konkrétně termooxidací. Stupeň oxidace materiálu katalyzovaný prooxidanty byl měřen pomocí FTIR. Nižšího karbonylového indexu, tj. nižšího stupně oxidace, bylo dosaženo v případě LDPE s plnivem, což bylo zřejmě způsobeno některým ze stabilizačních účinků plniva  $CaCO_3$ .

Jako doplňující experiment, potvrzující oxidaci materiálu, bylo provedeno měření prodloužení při přetržení. U obou vzorků bylo zjištěno zhoršení mechanických vlastností, tj. ztráta pevnosti.

K určení distribuční křivky molekulové hmotnosti polymerů byla užita gelová permeační chromatografie.

Vlastní biodegradace termálně oxidovaných PE fólií byla sledována v prostředí půdy a vyzrálého kompostu. Zkoumány byly především rozdíly v průběhu a rozsahu biodegradace mezi polymery s a bez obsahu plniva.

Přítomnost  $CaCO_3$  při biodegradaci v půdním prostředí mělo zanedbatelný vliv na biodegradabilitu LDPE fólií, což potvrzují také snímky získané ze skenovací elektronové mikroskopie, kdy na povrchu materiálu docházelo k tvorbě biofilmu.

Naopak, biodegradace v prostředí vyzrálého kompostu poukazovala na významně rychlejší mineralizaci LDPE bez plniva. Tento průběh mineralizace byl pravděpodobně ovlivněn vyšší teplotou při inkubaci, čímž mohla být způsobena snazší dostupnost biodegradabilních frakcí materiálu a projevil se vyšší stupeň oxidace materiálu bez plniva.

Účast a přítomnost mikrobiálního konsorcia na mineralizaci materiálu byla potvrzena technikou SEM. Tvorba husté sítě bakteriálních vláken byla charakteristická pro termofilní aktinobakterie.

Tato studie byla provedena ve spolupráci s kolegy z Centra polymerních systémů fakulty technologické Univerzity Tomáše Bati ve Zlíně. Autorka disertační práce měla na starosti biodegradační pokusy v komplexních prostředích tj. půdě a vyzrálém kompostu, podílela se rovněž na přípravě a redakci publikace.

## **5.3. Biodegradace PVA za denitrifikačních podmínek (Příloha P3)**

Schopnost biodegradace PVA za denitrifikačních podmínek byla sledována v prostředí aktivovaného kalu, který byl získán z centrální ČOV Zlín-Malenovice. Pro srovnání průběhu biodegradace byly experimenty provedeny také za podmínek aerobních. K získání informací o konkrétním degraderu tohoto polymeru bylo využito kombinace konvenčních a molekulárně biologických metod.

Počáteční experimenty inkubace polymeru s minerálním médiem a čerstvým aktivovaným kalem obohaceným o dusičnany (denitrifikační kal) nebyly úspěšné. Za těchto podmínek nebyla mikrobiální kultura téměř schopna PVA využívat jako zdroj uhlíku. Situace se změnila s obohacením čerstvého denitrifikačního kalu inokulem mikroorganismů z předešlého a ukončeného experimentu biodegradace PVA. Takto byl postupně získán tzv. adaptovaný kal s obohacenou mikrobiální komunitou. S užitím adaptovaného kalu byl

eliminován výskyt lagové fáze v počátku biodegradace PVA a mikrobiální konsorcium bylo schopno polymer hned využívat. Srovnáním křivek demonstrujících průběh biodegradace PVA za aerobních a anaerobních podmínek bylo konstatováno, že bakteriální komunita využívající polymer není na koncentraci kyslíku životně vázána, tj. tyto mikroorganismy lze zařadit do skupiny fakultativně anaerobních MO.

Izolace případných degradérů PVA nejprve proběhla konvenčním způsobem. Vzorky s obohacenou mikrobiální komunitou byly přeneseny na selektivní agar, nicméně se nepodařilo degradéra vyizolovat.

Užití molekulárně biologických metod bylo klíčovým bodem k izolaci a následné identifikaci druhu podílejícím se na mineralizaci PVA za denitrifikačních podmínek. Technikou TGGE, založenou na amplifikaci 16S rRNA genu V3 hypervariabilního regionu, byla zkoumána, v jednotlivých časových intervalech, dynamika biodegradace PVA. Analýzou TGGE profilů gelů byl vyselektován potenciální a dominantní degradér polyvinylalkoholu. K potvrzení převahy degradéra při biodegradaci, byla vytvořena DNA knihovna klonů genu 16S rDNA přítomných v konsorciu. Z 60-ti náhodně vyselektovaných klonů převládaly dva typy patřící do třídy Gammaproteobakterií. Jednalo se o druh *Steroidobacter* sp. ZUMI 37 a *Dokdanella* sp. LM.

Fylogenetická analýza získaných sekvencí z TGGE gelů potvrdila příbuzenské vztahy mezi *Steroidobacterem* sp. ZUMI 37, aerobním PVA degradérem izolovaným z půdy, a sekvencí získanou z obohaceného konsorcia v naší laboratoři. Předpokládaný degradér polyvinylalkoholu za denitrifikačních podmínek byl označen *Steroidobacter* sp. DP.

Kvantifikace zastoupení sekvence *Steroidobacter* sp. DP ve vzorcích v průběhu degradace byla provedena pomocí techniky qPCR. Experimenty potvrdily skutečnost, že množství části sekvence odpovídající bakteriálnímu rodu *Steroidobacter* sp. DP rostla se spotřebou polyvinylalkoholu.

Závěrem lze konstatovat, že PVA představuje polymer podléhající biodegradaci za denitrifikačních podmínek.

Studie byla vytvořena za účasti spolupracovníků na fakultě technologické Univerzity Tomáše Bati ve Zlíně a s pomocí kolegů z přírodovědecké fakulty Univerzity Karlovy v Praze. Autorka disertační práce spolupracovala na uskutečnění biodegradačních pokusů a samostatně prováděla molekulárně biologické metodiky (tj. TGGE, klonování, související práce) vedoucí k identifikaci degradéra.

#### **5.4. Vliv molekulové hmotnosti a formy PLA na její biodegradaci (Příloha P4)**

V této studii byla sledována mikrobiologie biodegradace PLA v závislosti na její formě a distribuci molekulových hmotností pomocí zvolených molekulárně biologických metod.

Biodegradačním testům v kompostu byly podrobeny čtyři různé vzorky PLA s molekulovou hmotností v rozsahu 34 000-160 000 g/mol<sup>-1</sup> ve formě jemného prášku, fólie a povlaku na inertním materiálu. Z křivek popisujících průběh biodegradace je patrné počáteční zpomalení odbourávání polymeru v případě vysokomolekulárních PLA (109 000 g/mol<sup>-1</sup>, 160 000 g/mol<sup>-1</sup>). Naopak lagová fáze zcela chyběla u nízkomolekulární PLA (34 000 g/mol<sup>-1</sup>) a středněmolekulární PLA (61 000 g/mol<sup>-1</sup>).

Stejně jako molekulová hmotnost, tak forma PLA ovlivňovala biodegradační pokusy. Zajímavým zjištěním byla skutečnost, že vyšší specifický povrch prášku nehrál významnou roli v případě nejvyšší molekulové hmotnosti a biodegradace byla téměř totožná s mineralizací PLA fólií. Jinak tomu bylo u vzorků PLA připravených jako tenký povlak na perlitu. Zde byl zaznamenán nejrychlejší průběh biodegradace u všech vzorků a u vysokomolekulárních PLA byla významně zkrácena doba počátečního zpomalení biodegradace.

Pro určení složení mikrobiálního společenstva účastnicího se biodegradace kyseliny polylactoné byla aplikována technika TGGE. Získané profily TGGE gelů odkryly jak signály bakteriálního konsorcia účastnicího se biodegradace PLA, tak signály pouze specifické komunity obsahující aktinomycey. Zástupci této významné skupiny gram pozitivních bakterií jsou často spojováni s mineralizací polymerů v kompostu a jejich účast byla potvrzena také v případě biodegradace PLA. Gelová elektroforéza v teplotním gradientu neodhalila významné rozdíly v mikrobiálním společenství v závislosti na různé molekulové hmotnosti. Naopak bylo zjištěno, že všechny zkoumané vzorky (s ohledem na různou molekulovou hmotnost a formu) byly degradovány stejným mikroorganismem.

Pomocí mikrobiální kultivace byly získány kolonie čistého kmene rostoucího na minerálním médiu se suspenzí PLA jako jediným zdrojem uhlíku a energie. Sekvenací 16S rRNA genu mikroorganismu a srovnáním získané sekvence s veřejnou databází Genbank byl vyizolovaný čistý kmen s 98% pravděpodobností shodný s bakterií *Thermopolyspora flexuosa*. Pomocí TGGE a sekvenční analýzy byla prokázána shoda mezi identifikovaným druhem mikroorganismu z minerálního média a signálem mikroorganismu (band na TGGE profilu) zodpovědným za biodegradaci PLA. Druh identifikován pomocí TGGE analýzy byl označen jako *Thermopolyspora flexuosa* FTPLA a je považován za klíčový organismus podílející se na biodegradaci PLA.

Studie byla uskutečněna ve spolupráci s kolegy na Univerzitě Tomáše Bati ve Zlíně, předkladatelka disertační práce se podílela na biodegradčních experimentech, studiu zúčastněných mikroorganismů pomocí zvolených molekulárně biologických metod a rovněž se podílela na přípravě a redakci publikace.

## 6. ZÁVĚR

Disertační práce se zabývala zkoumáním biodegradability vybraných polymerních materiálů s využitím kombinace konvenčních a molekulárně biologických metod. Biodegradační experimenty byly uskutečňovány v různých prostředích, tj. půdě, kompostu, a v prostředí vodném. Schopnost podléhat biodegradaci byla zkoumána konkrétně u PE, PVA a PLA.

Během doktorského studia vznikly čtyři odborné publikace (jedna z nich zatím není přijata), které mohou být rozděleny do dvou celků, vzhledem ke kombinaci použitých metod a materiálů.

V první části, obsahující dvě publikace, byla zkoumána biodegradabilita polyethylenových fólií obsahující prooxidanty pomocí konvenčních metod. V druhé části, obsahující rovněž dvě publikace, byl průběh biodegradace sledován s kombinací konvenčních a vybraných molekulárně biologických metod.

Konkrétně byly v experimentech popsanych v první publikaci studiu podrobeny různé PE fólie (HDPE, LDPE, LLDPE) obsahující rovnovážné množství antioxidantů a prooxidantů ( $Mn^{2+}$ ,  $Fe^{3+}$ ,  $Co^{2+}$ ). Jednotlivé vzorky byly abioticky předupraveny fotooxidací a termooxidací, což vedlo ke stárnutí polymeru, křehnutí materiálu a tvorbě nízkomolekulárních produktů. Schopnost biodegradace zkoumaných polymerů byla sledována jednak v prostředí minerálního média za účasti čistého kmene bakterie *Rhodococcus rhodochrous*, ale také v prostředí půdy a kompostu. Stanovením množství ATP bylo zjištěno, že povaha prooxidantů je hlavním faktorem ovlivňující biodegradaci PE fólií. Bakterie dokázaly asimilovat téměř všechny zkoumané PE fólie, vyjma těch s vysokým obsahem  $CO_2^{2+}$ . Pomocí techniky SEM byla potvrzena shoda mezi tvorbou biofilmu na povrchu polymeru a schopností polymeru podléhat biodegradaci.

Druhá studie měla za hlavní cíl prozkoumat vliv plniva, tj. mletého uhličitanu vápenatého v PE matrici, na průběh biodegradačních procesů v půdě a kompostu s využitím konvenčních metod. Srovnávány byly mezi sebou vzorky LDPE obsahující prooxidanty ( $Mn^{2+}$ ), jeden z nich navíc obsahoval  $CaCO_3$  jako plnivo. Stupeň oxidace abioticky termooxidovaných vzorků byl měřen pomocí FTIR. V případě LDPE s plnivem, bylo dosaženo nižšího karbonylového indexu, tj. nižšího stupně oxidace. Přítomnost plniva při biodegradaci v půdě měla zanedbatelný vliv na biodegradabilitu LDPE fólií. Naopak, významně rychlejší mineralizace LDPE fólií byla zaznamenána u vzorků bez plniva v prostředí vyžralého kompostu. Zde se projevil vyšší stupeň oxidace materiálu. Oba typy vzorků byly prozkoumány skenovací elektronovou mikroskopií, která potvrdila přítomnost bakterií na jejich povrchu. Jednalo se o termofilní aktinobakterie.

Účelem třetí studie, zabývající se schopností biodegradace PVA za denitrifikačních podmínek v prostředí aktivovaného kalu, byla identifikace konkrétního degradéra PVA především pomocí vybraných molekulárně biologických metod. Konvenční způsoby izolace případných degradérů na selektivním agaru se nezdařily. Užití molekulárně biologických metod bylo klíčovým bodem k identifikaci druhu podléjícím se na utilizaci PVA za denitrifikačních podmínek. Analýzou TGGE profilů gelů byl vyselektován potenciální a dominantní degradér PVA a jeho potvrzení převahy při biodegradaci bylo provedeno pomocí DNA knihovny klonů genu 16S rDNA přítomných v konsorciu. Fylogenetická analýza získaných sekvencí z TGGE gelů potvrdila příbuzenské vztahy mezi aerobním PVA degradérem *Steroidobacterem* sp. ZUMI 37 a sekvencí získanou z konsorcia v naší laboratoři. Degradér PVA byl námi označen jako *Steroidobacter* sp. DP. Technikou qPCR bylo kvantifikováno zastoupení sekvence *Steroidobacter* sp. DP ve vzorcích v průběhu biodegradace a byla potvrzena skutečnost, že množství části sekvence odpovídající

bakteriálnímu rodu *Steroidobacter* sp. DP rostla se spotřebou PVA. Polyvinylalkohol představuje syntetický polymer podléhající biodegradaci za denitrifikačních podmínek.

Závěrečná studie, v této disertační práci, se zabývala vlivem molekulové hmotnosti a formy kyseliny polyléčné na její biodegradaci. Mikrobiologie biodegradace PLA byla sledována převážně pomocí molekulárně biologických metod. Biodegradční testy probíhaly v prostředí kompostu a byly jim podrobeny čtyři různé vzorky PLA s různou molekulovou hmotností ( $34\ 000\text{--}160\ 000\ \text{g}\cdot\text{mol}^{-1}$ ) ve formě jemného prášku, fólie a povlaku na inertním materiálu. Stejně jako molekulová hmotnost, tak forma PLA ovlivňovala biodegradční pokusy. Bez lagové fáze byly mineralizovány vzorky nízkomolekulární a středněmolekulární PLA. Nejrychlejší průběh biodegradace byl zaznamenán u vzorků PLA připravených jako tenký povlak na perlitu. Technika TGGE byla aplikována pro určení složení mikrobiálního společenstva účastnícího se biodegradace PLA. Bylo zjištěno, že všechny zkoumané vzorky, jak s různou molekulovou hmotností, tak formou, byly degradovány stejným mikroorganismem. Pomocí TGGE a sekvenční analýzy byla prokázána shoda mezi identifikovaným druhem mikroorganismu z minerálního média a signálem mikroorganismu zodpovědným za biodegradaci PLA. Klíčový organismus podílející se na utilizaci PLA byl námi označen jako *Thermopolyspora flexuosa* FTPLA.

Během doktorského studia byly použity metody převzaté z literatury, zdokonaleny stávající metody, ale také zavedeny nové, které se ukázaly jako prospěšné. Konkrétně se podařilo modifikovat a zdokonalit metodu měření  $\text{CO}_2$  v případě biodegradčních experimentů. Vyřešeny byly problémy s netěsností biometrických lahví, kdy byl aplikován nový typ zátky, který zvýšil těsnost láhve. Komplikace při odběru vzorků pro plynovou chromatografii byly odstraněny použitím vhodnějšího typu plynotěsné stříkačky a jehly.

Nově zavedenou molekulárně biologickou metodou v naší laboratoři byla gelová elektroforéza v teplotním gradientu. Nejprve jsme museli překonat počáteční technické překážky u souvisejících prací, zvláště při přípravě PCR směsí bylo nutno předejít kontaminaci, kterou způsobovaly všudypřítomné bakterie. Modifikována byla samotná technika PCR, stejně tak jako složení a příprava TGGE gelů. Odstraněny byly problémy s tvorbou krystalků solí v matrici polyakrylamidového gelu a přilnavost gelů k polybond fólii. Byla vyřešena otázka s dostatečným průchodem elektrického proudu, nanášením vzorků na gel, barvením gelů, bobtnáním a scvrkáváním gelu, DNA extrakcí, reamplifikací a přípravou sekvenačních směsí. Pro doplnění výsledků získaných z na sebe navazujících molekulárně biologických metod byly modifikovány techniky pro vytvoření knihovny klonů vybraných genů z bakteriální komunity podílející se na biodegradčních procesech PVA.



## 7. CURRICULUM VITAE

### Osobní údaje:

Jméno a příjmení: Ing. Lucie Husárová  
Datum narození: 28. 2. 1985  
Adresa: Sídliště I/860, Nivnice, 687 51  
e-mail: [husarova.lucie@gmail.com](mailto:husarova.lucie@gmail.com)

### Vzdělání:

2009-doposud Univerzita Tomáše Bati ve Zlíně, fakulta technologická, navazující doktorské studium oboru Technologie makromolekulárních látek

2007 - 2009 Univerzita Tomáše Bati ve Zlíně, fakulta technologická, navazující magisterské studium oboru Inženýrství ochrany životního prostředí

2004 - 2007 Univerzita Tomáše Bati ve Zlíně, fakulta technologická, bakalářské studium oboru Chemie a technologie materiálů

2000 - 2004 SPŠ a VOŠ potravinářské technologie Bzenec, obor: Analýza potravin

### Účast na grantových projektech:

MSM 7088352101

Multifunkční kompozitní soustavy na bázi přírodních a syntetických polymerů (2005-2011, MSM)

GAČR No. P/108/10/0200

Biodegradation study of polymeric materials by combination of advanced methods (2010-2012).

IGA/FT/2012/027

Monitoring výskytu biogenních aminů ve vybraných potravinách a dekarboxylázové aktivity potravinářsky významných bakterií

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## 10. SEZNAM OBRÁZKŮ

Obr. 1 Schematické znázornění vlivu krystalizace polymeru na enzymatickou biodegradaci. Mikrobiální enzymy jsou schopny rozkládat amorfni podíl polymeru a hloubka biodegradace je ovlivněna přítomností krystalického podílu (upraveno podle [10]).....	7
Obr. 2 Zjednodušený mechanismus enzymatické biodegradace polymerů (upraveno podle [9]) .....	8
Obr. 3 Vzorec PVA .....	9
Obr. 4 Schéma na sebe navazujících kroků experimentu od izolace DNA z environmentálního vzorku, po konečnou identifikaci fragmentu DNA z TGGE gelu .....	12
Obr. 5 Výsledný paralelní TGGE gel z procesu biodegradace polymerního materiálu. Dráhy 1-6 představují vzorky odebrané v daných dnech biodegradace. Jednotlivé bandy zobrazují signály dominantních druhů mikroorganismů během procesu. V horní části gelu je patrné zesilování intenzity bandu, což poukazuje na množeni určitého MO, který využívá polymer pro svůj růst. ....	14
Obr. 6 Princip nested PCR.....	15
Obr. 7 Fylogenetický strom sekvencí získaných z TGGE analýzy .....	16
Obr.8 Biometrická láhev pro sledování míry biodegradace polymerů, která je opatřena uzávěrem se septem pro odběr vzorku plynné fáze a dvěma dalšími otvory pro provzdušňování.....	18
Obr. 9 TGGE Maxi Systém pro separaci PCR ampliconů.....	20

## 11. SEZNAM PŘÍLOH

Příloha P1: Srovnání biodegradability různých PE fólií obsahující prooxidanty.....	37
Příloha P2: Aerobní biodegradace polyethylenových fólií obsahující prooxidanty a uhličitan vápenatý jako plnivo.....	48
Příloha P3: Biodegradace PVA za denitrifikačních podmínek .....	54
Příloha P4: Vliv molekulové hmotnosti a formy PLA na její biodegradaci (Manuscript).....	62
Příloha P5: Use of temperature gradient gel electrophoresis for the investigation of poly(vinylalcohol) biodegradation (plnotextový příspěvek na konferenci) .....	77
Příloha P6: Identification of several factors affecting biodegradation of aromatic-aliphatic copolyester (plnotextový příspěvek na konferenci) .....	80
Příloha P7: Preparation of submicroparticles based on biodegradable co-polyester (plnotextový příspěvek na konferenci) .....	84
Příloha P8: Influence of long term storage on thermooxidation behavior of prooxidant containing low density polyethylene (plnotextový příspěvek na konferenci).....	90

# Příloha P 1: Srovnání biodegradability různých PE fólií obsahující prooxidanty

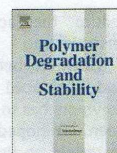
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## Comparison of the biodegradability of various polyethylene films containing pro-oxidant additives

Stéphane Fontanella<sup>a,b</sup>, Sylvie Bonhomme<sup>a</sup>, Marek Koutny<sup>c</sup>, Lucie Husarova<sup>c</sup>, Jean-Michel Brusson<sup>e</sup>, Jean-Paul Courdavault<sup>i</sup>, Silvio Pitteri<sup>f</sup>, Guy Samuel<sup>g</sup>, Gérard Pichon<sup>h</sup>, Jacques Lemaire<sup>a</sup>, Anne-Marie Delort<sup>b,d,\*</sup>

<sup>a</sup>Centre National d'Evaluation de Photoprotection (CNEP), Université Blaise Pascal, 63 177 Aubière cedex, France

<sup>b</sup>Clermont Université, UBP, Laboratoire SEESIB, F-63000 Clermont-Ferrand, France

<sup>c</sup>Tomáš Bata University in Zlín, Faculty of Technology, Department of Environmental Engineering, 762 72 Zlín, Czech Republic

<sup>d</sup>CNRS, UMR 6504, F-63177 Aubière, France

<sup>e</sup>Total Petrochemicals Research Feluy, Zone industrielle C, B-7181 Feluy, Belgium

<sup>f</sup>Basell Poliolefine Italia srl, G. Natta R & D Research Centre, Ple Donegani 12, 44100 Ferrara, Italy

<sup>g</sup>Association NEOSAC, F-43600 Ste Sigolène, France

<sup>h</sup>Groupe Extrusion Soufflage de Ste Sigolène (E3S), F-43600 Ste Sigolène, France

<sup>i</sup>Formerly employee of LyondellBasell, France

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### ABSTRACT

The biodegradability of high density polyethylene films (HDPE), low density polyethylene films (LDPE) and linear low density polyethylene films (LLDPE) with a balanced content of antioxidants and pro-oxidants (manganese + iron or manganese + iron + cobalt) was studied. Abiotic pre-treatment consisting of photooxidation and thermal oxidation corresponding to about three years of outdoor weathering (including 3–4 months of exposure to daylight) was monitored by FTIR and SEC measurements. The oxidized samples were then inoculated with the strain *Rhodococcus rhodochrous* in mineral medium, and incubated up to 180 days. The metabolic activity of the bacteria was assessed by measuring adenosine triphosphate content (ATP) and the viability of the cells. Complementary experiments were performed by <sup>1</sup>H NMR spectroscopy to monitor the biodegradation of soluble molecules excreted from the polymer in the incubation medium. Finally SEM was used to visualize the formation of a biofilm at the surface of the polymer. Three samples among the 12 tested were investigated in compost and soil environments. The results show that the main factor controlling the biodegradability of the polyethylene films is the nature of the pro-oxidant additive and to a lesser extent that of the matrix. Except for the samples containing very high content of cobalt additive, the various polymer films were used as substrates by the bacteria.

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### 1. Introduction

Increasing waste disposal problems from polymer packaging materials have resulted in constant endeavours to replace inert and non-biodegradable materials by biodegradable alternatives. Materials scientists have created new compositions and blends of known biodegradable materials, and invented new principles and ideas, in order to transform existing conventional materials into biodegradable ones. In this context polyethylene films containing pro-oxidants,

basically transition metal complexes inducing photo or thermal oxidation, are manufactured. The fine balance of antioxidant and pro-oxidant contents guarantees that after the preset period of service life, relatively fast abiotic oxidation begins. As a consequence, the material loses its mechanical properties and disintegrates into small fragments. These features provide a perspective solution to the problems of "visual pollution" by plastic litter that are constantly in the centre of public attention, although it may open a new problem of a medium-long term pollution by micro-fragments in the environment. On the molecular level the abiotic oxidation results in polymer chain fragmentation dramatic reduction of molecular weight, introduction of polar groups and increase of hydrophilicity. Such an oxidation process is thought to make the material much more vulnerable to microbial attack, which in the longer term could then

\* Corresponding author at: CNRS, UMR 6504, F-63177 Aubière, France. Tel.: +33 473 40 77 14; fax: +33 473 40 77 17.

E-mail address: A-Marie.DELORT@univ-bpclermont.fr (A.-M. Delort).



reduce the accumulation of such micro-fragments in the environment.

However, before being considered as eco-compatible products and introduced in the market, the biodegradability of such new polymers has to be tested. Basically two types of approaches have been developed to test the biodegradability of these specific materials; as already described in details by Koutny et al. [1] and Koutny and Delort [2].

First, the biodegradability of pro-oxidant activated PE can be evaluated in complex media like soil, waste water sludge or compost. The approach allows using a diverse microbial inoculum and apparently close relations to the real conditions in the nature and/or in waste treatment processes. This approach was used by Chiellini et al. [3] who showed 50–60% and 80% mineralization of a pre-oxidized film incubated under soil or compost conditions, respectively, after approximately one and a half year of incubation. Jakubowicz [4] claimed even 60% mineralization during only six month incubation. Quite recently, Ojeda et al. [5] reported about 12% mineralization after 90 days in compost.

A second approach is to use controlled experimental conditions, i.e. experiments with identified microbial strains in a medium formulated from defined chemical compounds. This type of experiments allows a better understanding of the process fundamentals, while it could also become the basis of standardised, easy to reproduce tests. Biodegradation with defined microbial strains was evaluated in several studies, most recently with *Pseudomonas aeruginosa* strain [6,7]. The authors brought some rather qualitative evidences that bacteria used some substances from polyethylene to support their metabolism. In a previous paper [8] we have proposed a new methodology to assess the biodegradability of polyethylene films containing pro-oxidants. Thermo and photo-oxidized films were incubated in mineral media containing the polymer as sole carbon source in the presence of pure microbial strains previously tested for oxidized PE degradation [9,10]. To follow the growth and development of microbial cultures the method for determination of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) was used. ATP is the key molecule of all living cell energetic metabolism. Its level reflects metabolic activity of a culture. This test method is extremely sensitive and quantitative, and it allowed us to show that the cell populations in presence of the oxidized PE samples were in a better energetic state than the control cultures that did not contain any polymer. It suggested that the cells were able to grow and to maintain their energetic status over months.

In this work, we decided to use this ATP test to assess the biodegradability of a large number of oxidized PE films. Three types of PE matrices were used, namely high density polyethylene films (HDPE), low density polyethylene films (LDPE) and linear low density polyethylene films (LLDPE), and three different pro-oxidant additives were compared: P1 complex (manganese + iron), P2 complex (manganese + iron) and P3 complex (manganese + iron + cobalt). The exact composition of these complexes is detailed in Table 1. Additive-free PE films served as reference samples.

The objective of the study was to compare these various films to determine which factors are more favourable for their biodegradability.

In addition to ATP content measurements, ADP content and cell viability were assayed. Complementary experiments were also performed by  $^1\text{H}$  NMR spectroscopy to monitor the biodegradation of soluble molecules extracted from the polymer into the incubation medium. Finally SEM was used to visualize the formation of biofilms at the surface of the polymer. Further, among the twelve samples tested, three of them have been incubated in soil and compost and  $\text{CO}_2$  produced during polymer biodegradation have been measured.

## 2. Materials and methods

### 2.1. Tested material

The material samples were transparent HDPE, LDPE and LLDPE films whose thickness ranged from 43 to 59  $\mu\text{m}$  (Table 1). The films contained iron photo-inducer supplying radicals through a photo-redox process and an organometallic type cobalt and/or manganese thermo-inducer, catalyzing the primary hydroperoxide decomposition (see Table 1). To antagonise the prodegradant activity of the photo- and thermo-inductor during the first year of storage and use under indoor conditions phenolic antioxidants were utilized in the blends. Due to the added antioxidants the thermal-induction period was longer than 400 h at 60 °C in the dark. The oxidation state of the catalysts was  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Fe}^{3+}$  and the ligand was stearate.

Elemental concentrations (wt%) of the different films were determined by X-Ray fluorescence on a Bruker S4 Pioneer spectrometer. Samples were measured under vacuum with excitation provided by a Rhodium X-ray tube and in a wavelength dispersive configuration with a 34 mm mask. Semi quantitative analysis was performed leading to a total concentration close to 100%, results are expressed as ratios of concentration of the detected element (Manganese, Iron, and Cobalt) to the total amount of carbon. A specific crystal (OVO-C) was used to measure this light element.

### 2.2. Abiotic treatment

The samples were exposed 10–40 h in the accelerated photo-ageing unit SEPAP 12.24 [9,11] ( $\lambda \geq 300$  nm, temperature of the exposed surface was set at  $60 \pm 1$  °C). After the exposure in SEPAP 12.24 the samples were considered sterile and treated in a sterile way. These 10–40 h of exposure corresponded to the photochemical induction period (total photo-transformation of phenolic antioxidants into inactive compounds).

Exposure in SEPAP was followed by 192–1224 h of treatment of the films in an aerated oven at 60 °C. Based on the activation energy determined when thermal oxidation proceeded (after the total consumption of phenolic antioxidants), i.e. the end of the total induction period, 300 h of thermal oxidation at 60 °C was equivalent to 2–3 years of thermal oxidation at room temperature in the dark (particles buried in the soil or particles not exposed to sunlight). The thermal treatment of additive-free films was extended to 2940–3912 h.

In the phase of true thermal oxidation beyond the induction period, the apparent activation energy was determined to be 100 kJ/mol, whereas in the phase corresponding to the thermal-induction period during which phenolic antioxidant counteracted the influence of the pro-oxidant additive, the apparent activation energy was only 40 kJ/mol.

The monitoring of the oxidation extent was carried out by transmission FTIR spectrophotometry with the non-fragmented films and by micro-FTIR spectrophotometry (FTIR spectrometer equipped with IR microscope) with the oxidized particles obtained after fragmentation.

### 2.3. Strains of bacteria

*Rhodococcus rhodochrous* ATCC 29672 was purchased from American Type Culture Collection.

### 2.4. Medium and conditions of cultivation

Mineral medium utilized throughout the study had the following composition: 3.8 g  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 1.8 g  $\text{KH}_2\text{PO}_4$ , 0.02 g



**Table 1**

Variation of the absorbance measured at  $\lambda = 1715 \text{ cm}^{-1}$  for different PE films containing or not pro-oxidant additives after abiotic treatment and metabolic and viability evaluation after incubation with *Rhodococcus rhodochrous* cells.

Additives	Ratio metal/carbon			Samples	Time of Exposure in SEPAP 12.24	Film thickness ( $\mu\text{m}$ )	Time of treatment in aerated oven $60^\circ\text{C}$	x/100	Absorbance increase at $1715 \text{ cm}^{-1}$	Viability test	[ADP]/[ATP]
	Fe	Mn	Co								
P1	$3.4 \times 10^{-4}$	$9.7 \times 10^{-4}$	0	LDPE	10 h	59	288 h	4.64	2.74	+	0.3
				HDPE	40 h	58	1224 h	2.22	1.29	+	1.0
				LLDPE	30 h	47	360 h	6.15	2.89	+	0.2
P2	$3.0 \times 10^{-4}$	$1.2 \times 10^{-3}$	0	LDPE	10 h	59	264 h	4.64	2.74	+	0.9
				HDPE	30 h	59	1056 h	1.76	1.04	+	1.9
				LLDPE	20 h	43	434 h	>7.0	>>3	+	0.8
P3	$4.0 \times 10^{-4}$	$1.1 \times 10^{-3}$	$3.2 \times 10^{-4}$	LDPE	10 h	59	192 h	>5.0	>>3	–	nd
				HDPE	40 h	59	456 h	3.81	2.25	–	5.8
				LLDPE	20 h	48	408 h	>6.0	>>3	–	4.9
None	0	0	0	LDPE	40 h	56	3720 h	0.45	0.25	nd	nd
				HDPE	40 h	59	3912 h	0.0	0.0	+	7.8
				LLDPE	20 h	52	2904 h	0.08	0.04	+	6.0

nd: not determined. (–) no growth in TS medium after 180 days, (+) growth in TS medium after 180 days. x is the thickness of the film in microns. When the absorbance measured at  $1715 \text{ cm}^{-1}$  is equal to x/100, PE fragmentation is observed.

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.03 g  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ , 0.01 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.5 g NaCl, 0.3 g  $\text{NH}_4\text{Cl}$  and 1 ml of trace element solution in litre. The trace element solution contained 0.20 g  $\text{MnSO}_4$ , 0.029 g,  $\text{H}_3\text{BO}_3$ , 0.022 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.0 g  $\text{Na}_2\text{MoO}_4$ , traces of  $\text{Co}(\text{NO}_3)_2$ , and traces of  $\text{CuSO}_4$  dissolved in 500 ml of water.

Thimerosal at final concentration 0.01% (w/w) was added into abiotic control cultivations as a growth inhibitor.

In general incubations for SEM, SEC and NMR spectroscopy observations were done in closed 100 ml glass flasks with 20 ml of media, incubation for ATP level determination in 4 ml closed glass vials with 0.4 ml of media. In both cases the head spaces were sufficiently large to provide the cultures with oxygen; moreover the flasks and vials were opened weekly so that the head-space air could be refreshed. Gas-tight sealing of the vessels was necessary to prevent water evaporation during the long incubation. The cultures were kept at  $27^\circ\text{C}$  with gently shaking. PE substrate concentrations were about 4 mg/ml for flask cultures and about 5 mg/ml for cultures in vials.

## 2.5. FTIR measurements

Small fragments whose section could be as small as  $1000 \mu\text{m}^2$  were analyzed by micro-FTIR spectrophotometry (NEXUS, Thermo Nicolet) with CONTINUUM microscope. The IR beam entering the spectrophotometer was focused on a predefined  $1000 \mu\text{m}^2$  zone of the small fragment and micro-FTIR spectrum was recorded in the transmission mode with a resolution of  $\pm 2 \text{ cm}^{-1}$  and absorbance defined with  $\pm 0.001$  precision.  $\mu$ -FTIR spectra were recorded on a Nicolet 800  $\mu$ -FTIR spectrometer connected to a NIC-PLAN IR microscope.

## 2.6. Size exclusion chromatography (SEC)

**HPDE samples:** A few milligrams of each sample were put in 10 ml vials, then the solvent 1,2,4-trichlorobenzene (from Sigma Aldrich, Saint Louis, Missouri, USA) was added. The solvent was stabilized with 0.025% by weight of 2,6-di-tert-butyl-4-methylphenol (BHT). A concentration of approximately 0.5 mg/ml was obtained. The vials were placed in a heating and shaking PL–SP 260VS plate (from Polymer Labs, Church Stretton, UK) at the temperature of  $160^\circ\text{C}$  for about 45 min. Once the dissolution had taken place, the vials were transferred into the carousel of the chromatograph autosampler. The solution was also filtered when some non-dissolved material appeared to remain in the vial after the heating step.

Molecular weight distributions were determined with high temperature GPC system Waters (Milford, Massachusetts, USA)

Alliance GPC2000 for polyethylene samples, equipped with a pre-column (Shodex UT-G) and separation columns Shodex UT 806 M (3 $\times$ ) and Shodex UT 807 and a PolymerChar (Valencia, Paterna 46980, Spain) IR-4 infrared detector as concentration detector. NTeq GPC software (hs GmbH, Hauptstraße 36, D-55437 Ober-Hilbersheim) was used for data acquisition and treatment.

The flow rate was 1.0 ml/min and the working temperature was  $145^\circ\text{C}$ . The injection volume was 400  $\mu\text{l}$ .

Solutions of polystyrene molecular weight standards were used for the calibration, according to the ISO 16014-2 method (Universal calibration).

**LPDE and LLPDE samples:** Solutions of 1,2,4-trichlorobenzene containing 0.1% at room temperature of oxidized LLDPE and LPDE films, and 1 g/l of BHT were prepared at  $150^\circ\text{C}$  for 1 h without agitation and filtered on a  $0.50 \mu\text{m}$  membrane using a Polymer Laboratories PL-SP260 apparatus. A volume of 400  $\mu\text{l}$  of this solution at room temperature was injected in the SEC apparatus (GPCV2K, Waters) equipped with three columns, 2 Shodex AT-806MS (Showa Denko) and 1 Styragel HT6E (Waters). The injector and column compartment were maintained at  $145^\circ\text{C}$  and the pump compartment at room temperature. The pump flow was 1 ml/min, the solvent was filtered on a  $0.45 \mu\text{m}$  membrane.

## 2.7. ATP and ADP assays

ATP Biomass Kit HS by Biothema (Sweden) was used for ATP determination. For each determination ATP from entire culture in 4 ml vial was extracted. At least three vials were analyzed for each time point. ADP was determined after transformation of ADP to ATP directly in the luminometer cuvette. Reaction mixture contained 30  $\mu\text{l}$  of the sample extract, 240  $\mu\text{l}$  of diluent B from the ATP kit, amended with 20 mM KCl and 2 mM  $\text{MgSO}_4$ , and 10  $\mu\text{l}$  of solution containing 0.1 M phosphoenolpyruvate and 5 mg/ml protein kinase in 0.05 M Tris–acetate buffer pH 7.2 [12]. The mixture was incubated 10 min at  $37^\circ\text{C}$ , 15 min equilibrated to the lab temperature and then the light producing reaction was started by addition of 60  $\mu\text{l}$  ATP reagent HS (Biothema), reconstituted with 2.5 ml of ATP free water. Blank experiment was done simultaneously to correct results for the background signal of the reagents.

## 2.8. Scanning electron microscopy

SEM micrographs were obtained using a ZEISS MEB Feg, type SUPRA 55VP.

Polymer samples were centrifuged in filtration tubes ( $0.2 \mu\text{m}$ ); fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) [13]



for 1–2 h at 4 °C, rinsed three times with 0.1 M phosphate buffer and finally dehydrated with a series of ethanol mixtures (50%, 70%, 95% and 100%) three times for 15 min at room temperature [14]. These samples placed in dried oven maintained at 27 °C for 30 min were mounted on aluminium stubs, sputtered with gold nanoparticles before SEM imaging.

### 2.9. NMR spectroscopy

NMR spectra were recorded after filtration of the media through 0.2 µm porosity filter.

NMR samples were prepared as follows: Supernatants (540 µL) resulting from biodegradation tests were supplemented with 60 µL of a 2 mM solution of TSPd<sub>4</sub> (sodium tetra deuterated trimethylsilyl propionate, Eurisotop) in D<sub>2</sub>O (Eurisotop). D<sub>2</sub>O was used for locking and shimming while TSPd<sub>4</sub> constituted a reference for chemical shifts (0 ppm) and quantification. <sup>1</sup>H NMR spectra were recorded at 400.13 MHz on a Bruker Avance 400 spectrometer at 21 °C with 5 mm-diameter tubes containing 600 µL of sample. 128 scans were collected (90° pulse, 6.84 s acquisition time, 1.0 s relaxation delay, 4789.272 Hz SW, 65536 data points). Water signal was eliminated by pre-saturation. An exponential filter was applied before Fourier transformation and a baseline correction was performed on spectra before integration with Bruker software. Under these conditions, the limit of quantification is in the range of 0.05 mM.

### 2.10. Biodegradation in compost and soil

Previously oxidized samples were disintegrated into fragments less than 2 mm, and mixed with mature compost (or fresh forest soil) and perlite in the ratio 1.5:2.5:10. All components were weighed into biometric flasks (1 g of oxidized polyethylene per flask) equipped with septa on stoppers and fitted with another two valves for aeration between measuring cycles. Sample flasks were incubated at 25 °C for biodegradation in the soil environment and at 58 °C for biodegradation under compost conditions. In the compost experiment presented here the temperature is, unlike in a real composting, artificially maintained at the preset value during the whole incubation period, so that conditions ideal for the intensive metabolism of thermophilic microorganisms are ensured. Head-space gas was sampled at appropriate intervals through the septum with a gas-tight syringe and then injected manually into a GC instrument (Agilent 7890). Sampling intervals were operatively adapted to actual CO<sub>2</sub> production and O<sub>2</sub> consumption. From the CO<sub>2</sub> concentration found, the percentage of mineralization with respect to the initial sample's carbon content was calculated. Endogenous production of CO<sub>2</sub> by soil or compost in blank incubations was always subtracted to obtain values representing net sample mineralization. In parallel, oxygen concentration was also monitored to provide a control mechanism so as to ensure samples did not suffer from hypoxia. Three parallel flasks were run for each sample, along with four blanks and positive control flasks, one with microcrystal cellulose as standard reference for hydro-biodegradable polymers and hexadecane as a reference with polyethylene like molecular structure, for both compost and soil incubations. Due to the technical problems during the test setup the LLDPE sample in compost was run in two parallel flasks only.

## 3. Results

### 3.1. The abiotic treatment

The following procedure was aimed at preparing samples corresponding to the material after weathering during the predefined

time period in outdoor conditions (see Section 2.2). After this period by the action of the pro-oxidants, light and heat, the material should be substantially chemically transformed, and thus more susceptible to a microbial attack.

To obtain the samples required for the experiments with microorganisms large areas of additive-containing HDPE, LDPE and LLDPE film were exposed briefly (10–40 h) in the SEPAP 12.24 photo-aging unit. The unit was originally designed for the exposure of small samples.

The pre-photo-oxidized films were transferred to a sterile box and submitted to thermal oxidation at 60 °C during 192–1224 h. At the end of the abiotic treatment the absorbance increase at 1712 cm<sup>-1</sup> determined by micro-FTIR spectroscopy varied from 1.04 to 3x/100 (where x was the film thickness).

As will be described in detail later, some substances can be released from the oxidized polymers to the water phase. Motivated by the concern for the washing out of pro-oxidant additives under humid or aquatic environment, some experiments were also carried out where the samples were exposed submersed in water in the SEPAP 12.24 H photo-aging unit. Similar results were obtained under these conditions compared to exposure under dry conditions, suggesting that the additives were not leached out to a significant extent.

#### 3.1.1. Assessment of oxidation extent in fragmented and non-fragmented films

As shown in Table 1, the additive-free LDPE, HDPE and LLDPE polymer films were slowly oxidized to a low extent, or even remained completely non-oxidized after 3912 h as in the case of HDPE. The short SEPAP 12.24 exposure was not able to completely convert the phenolic antioxidants into inactive compounds. In contrast (Table 1), the oxidation extent of the polymers with additives was much higher in the presence of pro-oxidant species than in their absence. The cobalt-P3 complexes induced the highest degree of oxidation for the 3 matrices examined. In the presence of every pro-oxidant additive, the HDPE matrix was the less sensitive to oxidation. This is likely due to the lower content of vinylidene defects which were shown to be directly correlated to the oxidized functions [15]. Also there are fewer tertiary carbons in HPDE material and its molecular weight is much higher (see Table 2).

In most PE films loss of 50% of mechanical properties was observed when absorbance increase at 1715 cm<sup>-1</sup> of acidic groups was equal to x/100, where the observed x is the thickness in microns of the oxidized film. Spontaneous fragmentation, i.e. fragmentation without the application of significant mechanical stress occurred when absorbance increase at 1715 cm<sup>-1</sup> was around x/100, depending on the oxidation mechanisms and on the film process conditions.

#### 3.1.2. SEC results

The average molecular weights of the polymers samples before and after abiotic treatments are presented in Table 2. After the abiotic treatment the Mn and Mw values of the oxidized polymers were much lower than those of the initial polymers. Clearly the presence of pro-oxidant additives enhanced the chain breakage of PE chains under thermo and photooxidation. The reduction of molecular weight Mn and Mw of the different polymers exposed to abiotic treatment is consistent with the degree of oxidation measured. For instance, in the case of the HPDE matrix, whatever the additives, Mw was higher than for the other matrices and the degree of oxidation always lower. Also, when comparing the nature of the pro-oxidant, the Co additive led to a higher degree of oxidation, whatever the matrix, and the Mw was relatively lower.



**Table 2**  
Average molecular weight of the polymers samples before and after the abiotic and biotic treatment.

Polymer	Samples	LDPE			HDPE			LLDPE		
		Mn	Mw	Ip	Mn	Mw	Ip	Mn	Mw	Ip
P1	Initial	16 500	89 822	5.4	9400	242 600	25.7	27 500	111 000	4.0
	After abiotic oxydation	2500	3756	1.5	1680	9850	5.9	2450	4000	1.6
	After biodegradation	2600	4117	1.6	760	8200	4.7	2500	4200	1.7
	Ref. without cells	2600	4149	1.6	1800	8670	4.7	2550	4400	1.7
P2	Initial	16000	84 956	5.3	9800	220 800	22.6	26 800	109 600	4.1
	Abiotic	2500	3828	1.5	1960	9700	5.0	2500	4100	1.6
	After biodegradation	2500	4040	1.6	1800	10 800	6.0	2600	4400	1.7
	Ref. without cells	2600	4029	1.6	1900	9300	4.9	2600	4500	1.7
P3	Initial	15 950	87 767	5.5	10 800	243 000	22.6	28 000	112 800	4.0
	Abiotic	2000	2625	1.3	990	4300	4.3	1800	2400	1.3
	After biodegradation	2000	2675	1.4	1000	4500	4.3	1800	2400	1.3
	Ref. without cells	2000	2685	1.4	1000	4300	4.31	1800	2400	1.3
None	Initial	16 000	89 215	5.5	7600	262 000	35.0	27 900	111 000	4.0
	Abiotic	5800	15 803	2.7	10 200	178 800	17.6	14 900	44 200	3.0
	After biodegradation	5900	15 706	2.7	9400	141 000	14.9	11 700	34 400	3.0
	Ref. without cells	6500	19 402	3.0	9800	147 700	15.1	14 400	45 000	3.1

M<sub>w</sub>, weight average molecular weight; M<sub>n</sub>, number average molecular weight; I<sub>p</sub>, polydispersity index (M<sub>w</sub>/M<sub>n</sub>).

### 3.2. Evaluation of the biodegradability of PE films by *R. rhodochrous* strain in mineral medium

All of the following experiments were done in a medium containing only necessary growth supporting mineral ions and where the tested material was present as the only source of carbon and energy so that no objection could be raised that the microorganisms were profiting from other media component than the tested material. Because in the previous studies [8–10] the bacterial strain *R. rhodochrous* a ATC 29672 was found to be the most active in terms of biofilm formation on the PE surface and metabolic activity on PE films, it was thus chosen for the evaluation of biodegradability in the present study. In addition, *Rhodococcus* genus is abundant in the environment, particularly in soils and was shown to be very active towards the higher molecular weight hydrocarbons [16].

#### 3.2.1. PE films containing P1 pro-oxidant (Mn + Fe)

In order to follow the evolution of the microorganism culture and its metabolic activity ATP content was determined in given time intervals, the results obtained for the three matrices tested are presented in Fig. 1a. For the three samples, the ATP concentration increased quickly after 4 days of incubation and then remained stable over time during 180 days although a small initial decrease was observed between 4 and 8 days in the case of LPDE before stabilization. In contrast cells incubated in the absence of polymer had a much lower amount of ATP. These experiments show that *R. rhodochrous* cells were able to use the three different polymer materials as a carbon source. It can be noted the ATP content reached after 4 days was much lower for cells incubated with HPDE film (ATP concentration about 7 pmol ml<sup>-1</sup>) than those incubated with LDPE and LLDPE films (ATP concentration about 20 pmol ml<sup>-1</sup>): this could be due to the lowest degree of oxidation of this film (see Table 1).

The metabolic state of the microbial population can be better characterized with the knowledge of the other adenylate nucleotide contents. ADP contents were determined in addition to ATP. The ADP/ATP ratio can be considered as a measure of the cell's energetic state. The lower is this ratio, the higher is the energetic state of the cells. We previously showed [8] that cultures of *R. rhodochrous* in rich complete Trypcase Soja medium at the end of the exponential growth phase, and therefore with a very high energetic state, had an ADP/ATP = 0.25 whereas cultures in the absence of polymer, with a low level of energy, had an ADP/ATP of about 6.0. In this study (Table 1), the ADP/ATP ratios obtained after

180 days of incubation with LDPE and LLDPE films were of 0.3 and 0.2 respectively, confirming the very good metabolic state of the cells. The value for the HDPE matrix is a bit higher (ADP/ATP = 1.0) but still bacteria show a high metabolic activity when incubated with this polymer.

To confirm the viability of the cells at the end of the incubation, the incubation medium was spread on Petri dishes containing a rich medium (Tripcase Soja); the three types of film bacteria could grow actively after 180 days (Table 1).

To explain the maintenance of the high metabolic state of the bacteria when incubated with the different polymers, we hypothesize that some soluble substances could be extracted by the aqueous medium from the oxidized PE film and subsequently consumed by bacteria.<sup>1</sup>H NMR spectra of the liquid phase of the incubation media in the presence or absence of *R. rhodochrous* with the various polymers were recorded at regular time intervals. Fig. 2 shows the results obtained in the case of LDPE films containing P1 additive. Fig. 2a shows the <sup>1</sup>H NMR spectra collected after 0, 90 and 180 days of incubation in the absence of bacteria, the observed NMR signals correspond to small molecules eluted from the polymer into the incubation medium. Many signals are present between 0.8 and 3.7 ppm, these chemical shifts are consistent with substituted or not CH and CH<sub>2</sub> groups. Other signals are present between 7.21 and 8.46 ppm, they are likely to correspond to molecules containing C=C bonds, carboxylic or carbonyl groups. Some specific signals can be easily assigned such as those of ethanol resonating at  $\delta = 3.67$  and 1.20 ppm, and that of formic acid resonating at  $\delta = 8.46$  ppm. All these small molecules are consistent with derivatives of short oxidized PE polyethylene fragments. Although most of these signals remain unassigned, the evolution of their integrals can be followed with time and can be used as a fingerprint to assess their quantitative evolution with time. Fig. 2c shows that most of integrals remained stable after 20 days of incubation, except that of the signal resonating at 7.21 ppm which was slowly decreasing with time and that of the signal resonating at 1.2 which was slowly increasing. The results obtained in the presence of bacteria in the incubation medium are quite different: i) first the signals resonating at  $\delta = 7.51, 7.21, 8.46,$  and 1.72 ppm are no longer present in <sup>1</sup>H NMR spectra (Fig. 2b); ii) the integrals of the signals have been decreased by a factor 10 in the presence of *Rhodococcus* cells (Fig. 2d) compared to those measured in the absence of bacteria (Fig. 2c). These results clearly show that *Rhodococcus* cells metabolize the soluble molecules eluted from the LDPE films containing P1 additive.



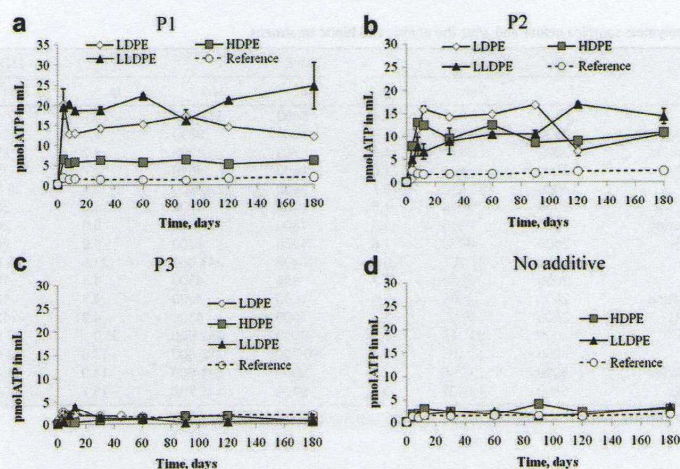


Fig. 1. Evolution of ATP content in *R. rhodochrous* cultures with oxidized LDPE films ( $\diamond$ ), HDPE films ( $\blacksquare$ ), LLDPE films ( $\blacktriangle$ ) and without polymer ( $\circ$ ), PE films containing P1 additive (a), P2 additive (b), P3 additive (c) and no additive (d).

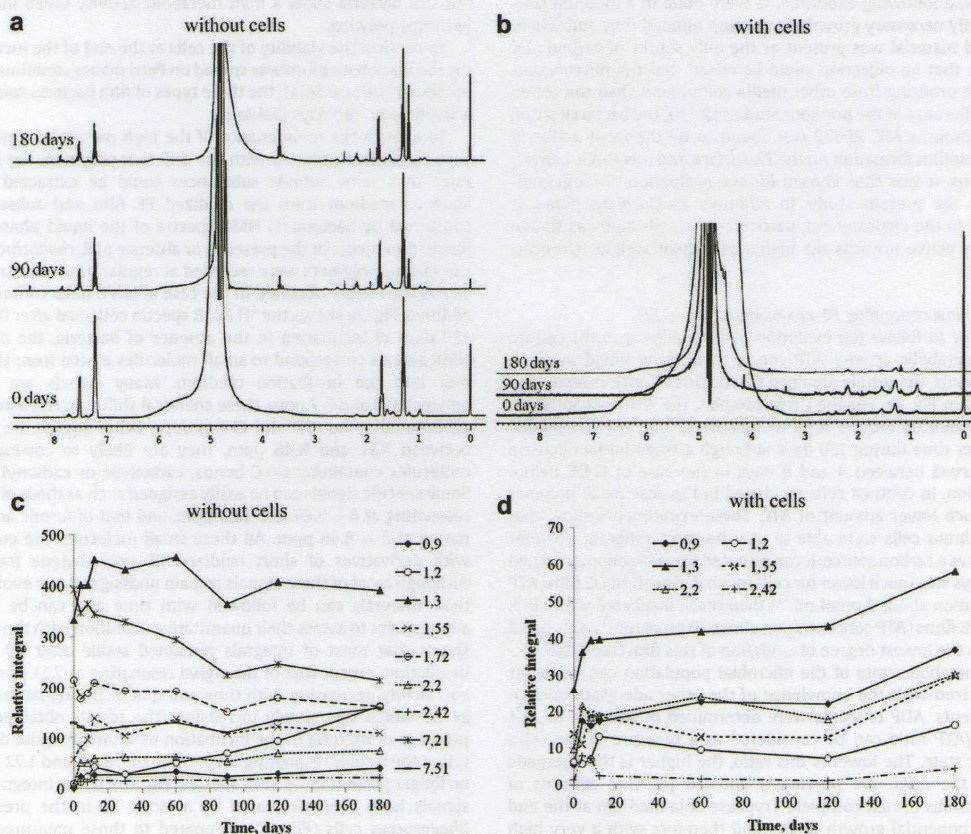


Fig. 2. Evolution with time of the soluble molecule content present in the incubation media of the LDPE film containing P1 additive.  $^1\text{H}$  NMR spectra were collected at various time of incubation in the absence (a), or the presence (b) of *R. rhodochrous*. The time courses of the integrals of NMR signals resonating at  $\delta = 0.9, 1.2, 1.3, 1.55, 1.72, 2.2, 2.42, 7.21$  and  $7.51$  ppm were plotted before (c) and after (d) incubation with *R. rhodochrous*.



In order to compare the different polymers and simplify the figures of the manuscript we have decided to take one of these signals ( $\delta = 1.3$  ppm) as its evolution is parallel to the other ones and can be used as a representative probe for these soluble molecules. Fig. 3a and b show the evolution of the signal at  $\delta = 1.3$  ppm for the three PE films additivated with P1 pro-oxidant, incubated without or with cells respectively. Clearly in the presence of cells the integrals of the signal at  $\delta = 1.3$  ppm are about ten times lower than after the incubation without cells, showing that the bacteria use these small molecules as a substrate to maintain a high energetic state. However HDPE film releases many fewer molecules than LDPE and LLDPE films (Fig. 3a) providing lower amount of substrates for the cells.

These results are fully consistent with the ATP and ADP/ATP values measured above. There are also consistent with the degree

of oxidation of the different films (see Table 1), the more oxidized is the polymer, the greater is the initial amount of molecules present in the aqueous phase.

Fig. 4 shows SEM pictures of the three different PE films additivated with P1 complex, at the end of the incubation with *R. rhodochrous* cells. A dense biofilm formation was observed at the surface of the LDPE and LLDPE polymer (Fig. 4a, c) while bacteria were more dispersed on HDPE film (Fig. 4b). This result also supports our previous conclusions (ATP and NMR results) showing that the HDPE film behaves differently than the two other matrices and was not so favourable for microbial metabolism. In the absence of cells, as expected no biofilm formation was observed (Fig. 4f).

Finally SEC experiments were performed on polymers at the end of the incubation with *R. rhodochrous* cells (Table 2). For any of the three materials the analysis did not reveal any significant difference

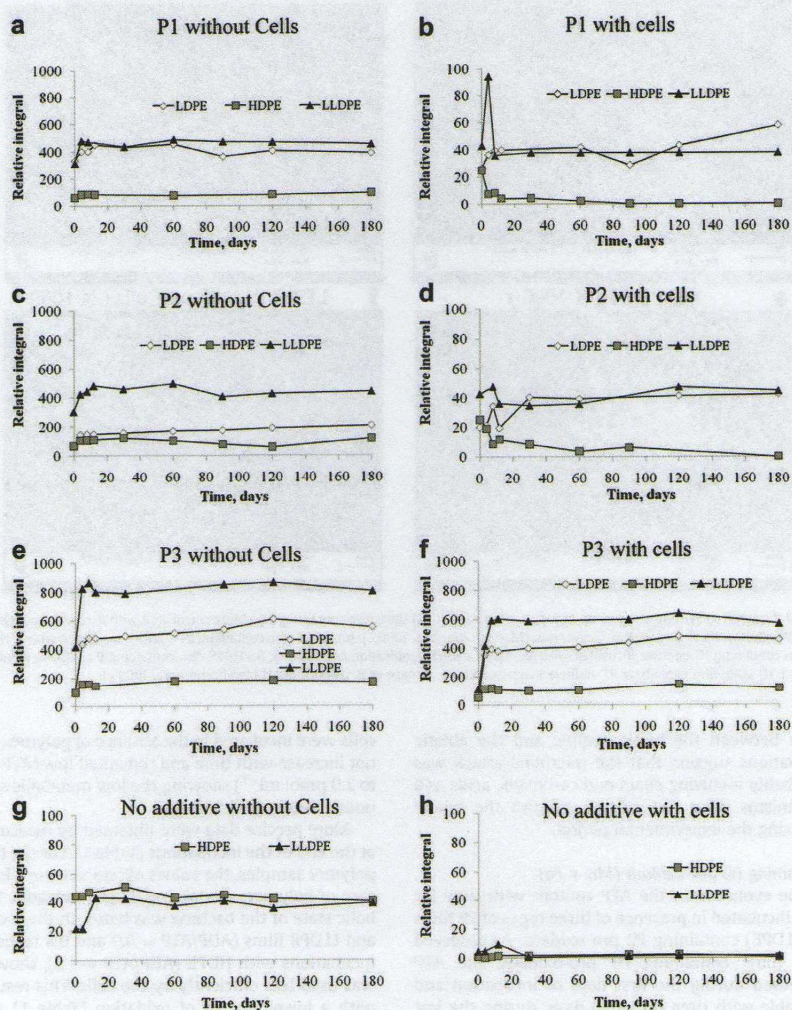
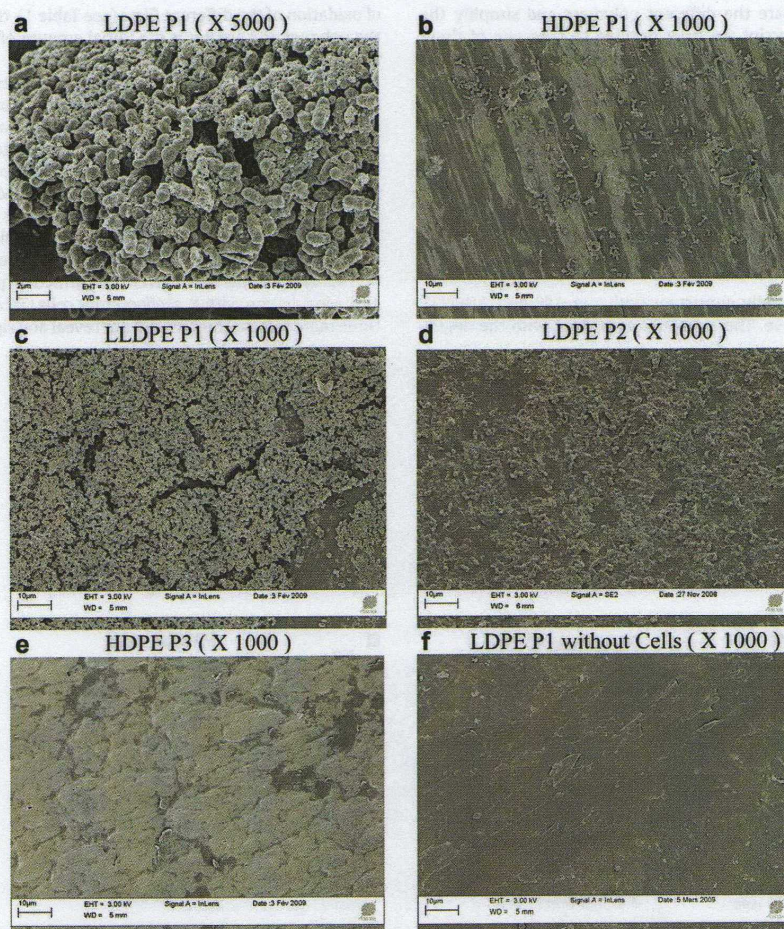


Fig. 3. Time courses of the integrals of the NMR signal resonating at  $\delta = 1.3$  ppm collected on incubation media with oxidized LDPE films ( $\diamond$ ), HDPE films ( $\blacksquare$ ) and LLDPE films ( $\blacktriangle$ ). PE films containing P1 additive incubated in the absence (a) or the presence (b) of *R. rhodochrous*. PE films containing P2 additive incubated in the absence (c) or the presence (d) of *R. rhodochrous*. PE films containing P3 additive incubated in the absence (e) or the presence (f) of *R. rhodochrous*. PE films containing no additive incubated in the absence (g) or the presence (h) of *R. rhodochrous*.





**Fig. 4.** SEM observation of the oxidized PE film surface after 180 days of incubation. (a) LDPE film containing P1 additive incubated with *R. rhodochrous* (Magnification cca. 5000 $\times$ ); (b) HDPE film containing P1 additive incubated with *R. rhodochrous* (Magnification cca. 1000 $\times$ ); (c) LLDPE film containing P1 additive incubated with *R. rhodochrous* (Magnification cca. 1000 $\times$ ); (d) LDPE film containing P2 additive incubated with *R. rhodochrous* (Magnification cca. 1000 $\times$ ); (e) HDPE film containing P3 additive incubated with *R. rhodochrous* (Magnification cca. 1000 $\times$ ); (f) LDPE film containing P1 additive incubated in the absence of *R. rhodochrous* (Magnification cca. 1000 $\times$ ).

in Mw distribution between the biotic sample and the abiotic control. The observations suggest that the microbial attack was only superficial probably involving chain end carboxylic acids and that the microorganisms were not able to perturb the whole material volume during the experimental period.

### 3.2.2. PE films containing P2 pro-oxidant (Mn + Fe)

Fig. 1b shows the evolution of the ATP content with time for *R. rhodochrous* cells incubated in presence of three types of PE films (LDPE, HDPE and LLDPE) containing P2 pro-oxidant. As observed previously for PE films containing P1 pro-oxidant, the ATP concentration increased during the first days of incubation and remained rather stable with time up to 90 days, during the last period it decreased during incubations with LDPE films, increased with LLDPE films and remained stable with HDPE films. The maximum ATP concentration value was around 16 pmol ml<sup>-1</sup> (Fig. 1b) and was reached within 12 days instead of 4 days. When

cells were incubated in the absence of polymer, the ATP content did not increase with time and remained low (ATP concentration close to 2.0 pmol ml<sup>-1</sup>) showing the low metabolic status of the bacteria under these conditions.

More precise data were obtained by measuring ADP/ATP ratios at the end of the incubations (Table 1). For the three P2 pro-oxidant polymer samples, the values obtained were a bit higher than in the case of polymers containing P1 pro-oxidants, but again the metabolic state of the bacteria was better in the incubations with LDPE and LLDPE films (ADP/ATP = 0.9 and 0.8 respectively) than in the incubations with HDPE (ADP/ATP = 1.9), showing this compound was used less efficiently by the cells. This result is also correlated with a lower degree of oxidation (Table 1) and the higher Mw measured after abiotic treatment (Table 2).

The metabolic activity of the cells was confirmed by their viability at the end of the experiments, growth was observed in Petri dishes for the three PE film incubations (Table 1).



$^1\text{H}$  NMR spectroscopy was used to monitor the amount of soluble molecules released in the culture medium and its evolution with time. In Fig. 3c and d are reported the time courses of the integrals of the signal resonating at  $\delta = 1.3$  ppm. As explained earlier, this signal is used as a representative probe for the rest of the signals. Clearly the amount of molecules released in media without cells (Fig. 3c) is much higher (factor 10) than that in culture media (Fig. 3d) showing the use of these molecules by the bacteria to maintain their energy status. This is in agreement with ATP measurements.

Observation by SEM of P2 additivated LDPE polymer film at the end of the incubations (Fig. 4d) showed that a biofilm was present on its surface, although it was less dense than in the case of LDPE film containing P1 additive (Fig. 4a). This result might be related to the relatively lower ATP content measured with LDPE films containing P2 pro-oxidant compared to those containing P1 pro-oxidant.

Finally SEC experiments showed no difference before and after biotic treatment for the three polymers (Table 2).

### 3.2.3. PE films containing P3 pro-oxidant (Mn + Fe + Co)

The same methodology was used to test the biodegradability of PE films containing P3 pro-oxidant, but quite different results were obtained with this additive compared to P1 and P2 additives.

First, the ATP content did not increase with time and remained very similar, whatever the matrix, to that observed in incubations without polymer (Fig. 1c), and the maximum ATP concentration value was less than  $4 \text{ pmol L}^{-1}$ .

This lack of energy was confirmed by the ADP/ATP ratio values (Table 1), the values obtained for the HPDE and LLDPE films were 5.8 and 4.9; i.e. very high values, reflecting a very low metabolic state of the bacteria. For technical reasons the ADP/ATP ratio could not be determined for the LDPE sample.

None of the cells present in these incubation media were able to grow on Petri dishes (Table 1), indicating that the cells were dead at the end of the experiments.

$^1\text{H}$  NMR spectra showed that actually a large amount of soluble molecules were present in the incubation media in the absence of cells; this is shown by the time courses of the reference signal at 1.3 ppm (Fig. 3e). This result is in agreement with the lower Mw measured by SEC experiments (Table 2). However, bacteria did not metabolize these organic compounds during the incubation as the integral of these signals remained stable with time (Fig. 3f). Because the strain and the soluble molecules are the same in the various experiments, we can conclude that bacteria do not lack substrate to maintain their metabolism but their metabolism was inhibited by this specific pro-oxidant containing cobalt. In addition the HDPE film containing P3 additive was not covered by any biofilm as shown by SEM observations (Fig. 4e). This negative result can be related to the low metabolic state and non viability of the bacteria when incubated with these materials.

In this case, SEC experiments again did not reveal any change in the presence of *Rhodococcus* cells.

### 3.2.4. PE films without pro-oxidant

In these experiments the tested films had similar matrices than in the sections 3.1, 3.2 and 3.3, namely LDPE, HDPE and LLDPE films, but did not contain any pro-oxidant additive.

Fig. 1d shows the evolution with time of the ATP content of *R. rhodochrous* cells incubated with the LLDPE and HDPE films (the LDPE film could not be studied for technical reasons). In all cases, the ATP concentration measured was quite low and close to what was observed in the absence of polymer. This result indicates that the energetic status of cells remains very low. ADP/ATP ratio values confirmed this information as the obtained values were 6.0 and 7.8 (Table 1). Bacteria present in the incubation media containing HDPE

and LLDPE films were still alive and could grow on Petri dishes (Table 1), showing that no toxic compound was present in the tested PE matrices.

$^1\text{H}$  NMR experiments showed that only few molecules were released into the incubation media (Fig. 3g), which is consistent with the very low oxidation degree and the higher Mw (Table 2) observed for these polymers due to the absence of pro-oxidants. These molecules were degraded by the cells (Fig. 3h), but the amount of carbon molecules was too low to maintain a high metabolic state of the cells, and bacteria could only survive under starving conditions.

SEC experiments did not show any difference before and after biotic treatment (Table 2).

### 3.3. Evaluation of the biodegradability of PE films in soil and compost

Among the twelve PE samples tested above using *Rhodococcus* strain in mineral medium, three have been tested in complex media, namely compost and soil that contain microbial consortia and complex organic carbon sources. The biodegradability was estimated by measuring  $\text{CO}_2$  production. Cellulose and hexadecane used as reference samples were tested in parallel. The three PE samples were chosen as follows: 2 samples were representative of those giving the "best results" with *Rhodococcus* cells, namely LDPE and LLDPE matrices containing P1 pro-oxidant; 1 sample was the one giving the "worst result", namely HDPE film containing P3 pro-oxidant.

The time courses of carbon mineralization of the various samples are reported in Fig. 5A and B for compost incubations and Fig. 5C and D for soil incubations.

As expected for easily degradable compounds, cellulose was efficiently degraded in compost; 100% degradation was reached after 140 days, while the percentage of hexadecane mineralization was close to 100% after 250 days. Values over 100% observed for cellulose at the end of the observation period can be explained by so called "priming effect" [17] and were observed also by other authors [5]. Although the biodegradation rate was much lower, LDPE and LLDPE films containing P1 complexes were bio-transformed in compost incubation; after 317 days the percentage of mineralization reached about 16% and 24% respectively. In contrast, for the HPDE sample containing P3 complex the mineralization reached less than 6%. Moreover, this value essentially corresponds to the initial burst of  $\text{CO}_2$  during first about 20 days of incubation and can be related to the consumption of easily biodegradable compounds possibly extracted into aqueous media. After this short period  $\text{CO}_2$  production nearly stopped. These results are fully consistent with the result described in Section 3.2 using *Rhodococcus* strain and mineral salt.

The same type of results was obtained in incubations with soil; although all the samples were mineralized much slower than in compost. The percentages of cellulose and hexadecane mineralization reached 69% and 47%, respectively after 352 days of incubation. Again the HPDE sample containing P3 pro-oxidant was mineralized to a significantly lower extent (<5%) while the LDPE and LLDPE samples containing P1 pro-oxidant reached 9% and 12%, respectively, and rates of their biodegradation were clearly positive in the end of the observation period.

The results obtained in both soil and compost are in complete agreement with the ADP/ATP ratios obtained for the samples incubated with *R. rhodochrous* (see Table 1).

## 4. Discussion and conclusions

In the present work, we have studied the potential biodegradability of PE films containing pro-oxidant additives that could be



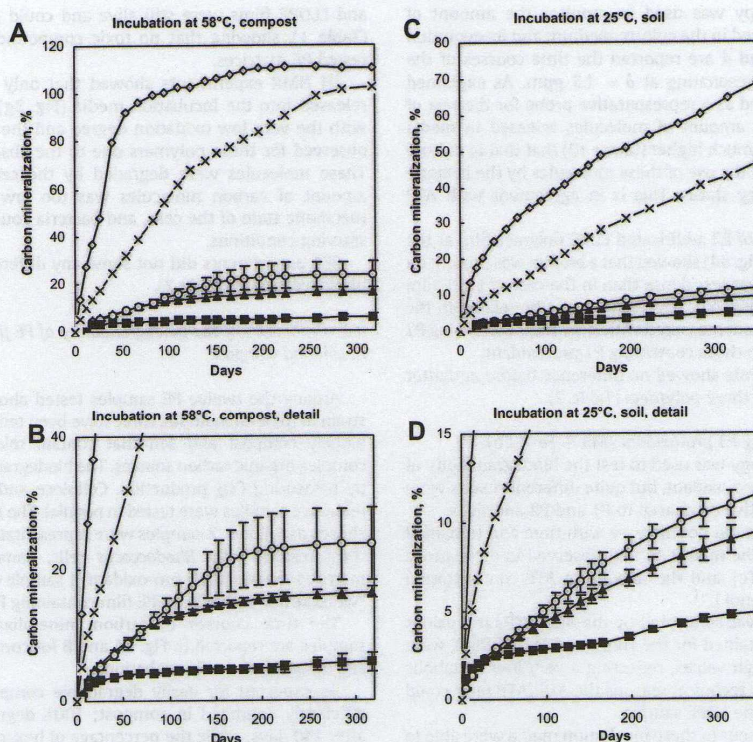


Fig. 5. Incubations in compost (A and B) and soil (C and D): % of carbon mineralization calculated from  $\text{CO}_2$  measurements. ▲, LDPE (P1); ○, LLDPE (P2); ■, HDPE (P3); ◇, cellulose (reference compound); ×, hexadecane (reference compound). Error bars correspond to twice standard deviation.

considered as part of a new branch of polymer materials with time-dependent mechanical properties. The objective was to compare the influence of the type of PE matrix (LDPE, HDPE and LLDPE) and the nature of the pro-oxidant additive (complexes of Mn + Fe with or without Co) on the ability to go through abiotic and biotic transformations.

The first important result is that, whatever the pro-oxidant used, the HDPE matrix is less efficiently oxidized than the other two types of PE. As a result the amount of low molecular weight molecules which are soluble in the incubation media and detected by  $^1\text{H}$  NMR spectroscopy and the molecular weight distribution measured by SEC experiments are directly proportional to this degree of oxidation. A lower amount of compounds is extracted from HDPE films compared to the others. This result confirms previously published data [8]. The clearest demonstration is given in the case of additive-free polymers which are poorly oxidized and consequently poorly transformed into small molecules as shown by  $^1\text{H}$  NMR and SEC measurements. Following this logic, these small molecules which were probably the low molecular weight degradation products of polyethylene chains most often terminated with carboxylic group already observed in other studies [18,19] can serve as carbon substrates for bacteria, participating in the biodegradation of the polymer. Again when only a limited amount of substrate is available, as in the case of additive-free PE polymers, and although bacteria remain alive after 180 days, their metabolic state is very low (ATP, ADP measurements) as a result of a low biodegradation activity. When soluble molecules resulting from the abiotic oxidation are readily available for bacteria, their metabolic state can be high even after a long period of incubation:

this is true for PE films containing P1 and P2 pro-oxidants but not for those containing P3; because bacteria are not substrate limited when incubated with PE films containing P3 pro-oxidant, it can be suggested that this additive inhibits the metabolism of *R. rhodochrous* while the other metal complexes do not. The second important result of this study is thus that the nature of the pro-oxidant additive is the major factor controlling the biodegradability of the PE film. In particular the relative amounts of metals in the complex is determinant. For example in this study P3 contains a combination of Mn, Fe and a high concentration of cobalt. This high concentration of Co seems to be a limiting factor since, as it was previously shown that HDPE and LDPE films containing a Co + Fe additive with lower Co content did not inhibit *R. rhodochrous* cells and could serve as carbon substrates for the cells during at least 200 days [8].

Cobalt is not toxic at low concentrations and can be used as pro-oxidant in PE films with limited and controlled concentrations.

The third important result deals with the link between the efficiency of polymer biodegradation and the formation of a biofilm at the polymer surface. It is important for bacteria to adhere to the PE film to be able to metabolize it. The more oxidized is the PE polymer, the more hydrophilic it becomes, and the highest is the interaction with *Rhodococcus* cells (except in the case of the high Co-content additivation, for which cells do not form any biofilm because they are basically dead). This result confirms previous data showing that *Rhodococcus* cells formed more dense and compact biofilms on the surface of oxidized LDPE films compared to less oxidized HDPE films [8]. *R. rhodochrous* was also shown to form biofilms on other oxidized PE polymers [10].



Finally, an important result is that experiments performed in compost and soil, which are more complex and less controlled but closer to environmental conditions, were absolutely consistent with the results obtained under our simplified conditions using one pure strain of *Rhodococcus* with the polymer film as sole carbon source and evaluating the biodegradability with the "ATP test", we developed earlier [8]. We reached the same conclusions concerning the influence of the pro-oxidant additives: while PE films containing P1 and P2 complexes were biodegraded under simplified or complex conditions, the PE film containing P3 complex was not. It also shows that these two tests are complementary and consistent; this work thus validates the pertinence of using the "ATP test". Also it underlines the interest of using simplified and highly controlled media and conditions as we were able to run NMR spectra and other complementary experiments that gave insights in the involved mechanisms. For instance the biodegradation activity was shown to be connected with the amount of small and soluble molecules, the degree of oxidation and the formation of a biofilm.

In conclusion, the data reported here show that *Rhodococcus* cells were able to remain metabolically active on at least six types of PE films containing pro-oxidants during 180 days, suggesting that they gain energy from the material, hence from an ongoing biodegradation. The nature of PE matrix is important but the most important parameter is the nature, the composition and the concentration of the metals present in the complexes used as pro-oxidant additives. This was observed both under laboratory conditions using the ATP test in mineral medium and *Rhodococcus* strain, but also under experiments performed in soil and compost. Although it is not possible to make even a rough estimation of the process extent and eventually the time necessary for the ultimate biodegradation of the sample under applied laboratory conditions, we believe that the results presented here allow to progress in the understanding and possibly the applications of the processes put into evidence. The combination of appropriate PE matrices and pro-oxidants could lead to manufacture biodegradable products for some applications, although it should be always kept in mind that this biodegradation will be at a slow rate, in particular when compared with the much faster rate of fragmentation caused by the polymer oxidation.

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## Příloha P 2: Aerobní biodegradace polyethylenových fólií obsahující prooxidanty a uhličitán vápenatý jako plnivo

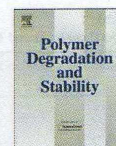
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### Aerobic biodegradation of calcium carbonate filled polyethylene film containing pro-oxidant additives

Lucie Husarova<sup>a</sup>, Michal Machovsky<sup>b</sup>, Pavel Gerych<sup>a</sup>, Josef Houser<sup>a</sup>, Marek Koutny<sup>a,\*</sup>

<sup>a</sup> Department of Environmental Protection Engineering, Faculty of Technology, Tomas Bata University in Zlin, 762 72 Zlin, Czech Republic  
<sup>b</sup> Polymer Centre, Faculty of Technology, Tomas Bata University in Zlin, 762 72 Zlin, Czech Republic

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#### ABSTRACT

Two low density polyethylene films, which contained pro-oxidant additives, where investigated, one of them containing micro-milled calcium carbonate filler. Both materials were subjected to controlled thermal oxidation, the oxidation was obviously retarded in the filler-containing sample. Following this, the biodegradability of samples pre-oxidized for 40 and 80 days was investigated. The levels of carbon mineralization reached 13% and 16% for the 40 and 80 days pre-oxidized polyethylene containing filler, respectively, after approximately 16 months in a soil environment at 25 °C, and both types of sample were mineralized to about 19% in compost environment at 58 °C during the same period. The sample not containing filler was mineralized to about 7% in soil after 13 months, and about 23%, after 8 months in compost. Scanning electron microscopy revealed dense colonization of the sample surfaces in both soil and compost. The data presented here provide clear quantitative evidence that part of the polyethylene material was biodegraded.

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#### 1. Introduction

In the past few decades a growing public aversion to common polymer wrapping materials has inspired many attempts to develop competitive biodegradable materials. One of them, originally dating from long prior to 1980 [1], was a trial to promote biodegradation in conventional polyolefin materials, especially polyethylene. The approach consisted of adding oxidation inducing additives, i.e. pro-oxidants, and was later shown to be very efficient when in soil the recorded extent of carbon mineralization during the biodegradation of LDPE film, which contained pro-oxidants, reached approximately 50% after 600 days [2], and even about 60% after 200 days in a laboratory composting unit [3]. However, such high mineralization results have never been reproduced and confirmed. Instead, later studies reported significantly lower [4] or merely limited [5,6] progress of biodegradation. Quite recently, Ojeda et al. [7] reported about 12% mineralization after 90 days in compost. In our laboratory, minimal carbon mineralization was repeatedly observed during biodegradation experiments with abiotically pre-oxidized polyethylene containing pro-oxidant. Nevertheless, significant rates of biodegradation were seen with several samples, reaching levels of mineralization of about 15% after

one year of incubation in soil or mature compost (results not published).

Presented here are results concerning a promising sample tested i.e. low density polyethylene film containing Mn ion based pro-oxidants filled with micro-milled calcium carbonate. Thermal oxidation of the material was previously investigated through a combination of FTIR and an innovative luminescence method, the latter bringing yet more direct evidence to support the conventional idea that metal ions in pro-oxidants act through catalysis of hydroperoxide decomposition [8]. This work examines the biodegradation of previously thermally-oxidized material in soil and compost environments. The same biodegradation experiments were also conducted with another LDPE film containing Mn based pro-oxidant additive, but without calcium carbonate filler. The two materials were characterized and found comparable except for the filler content (Table 1). Results from both materials were compared.

#### 2. Experimental

##### 2.1. Materials

The first material (LPa) used in the study was previously described and characterized in [8]. The sample was low density polyethylene Bralene RB 0323 as a semi-transparent film, 50–55 μm thick and containing Omyalen 2021 P (15% w/w) micro-milled lime

\* Corresponding author.  
E-mail address: [mkoutny@ft.utb.cz](mailto:mkoutny@ft.utb.cz) (M. Koutny).



**Table 1**  
Metal contents and MW changes during thermal oxidation at 70 °C.

Sample	Mn content µg g <sup>-1</sup>	Fe content µg g <sup>-1</sup>	CaCO <sub>3</sub> filler % w/w	Mn	Mw	Mw/Mn
LPa	60.0	70.6	15	24 600	293 400	12.1
LPa40	—	—	—	3900	33 300	8.43
LPa80	—	—	—	4100	8600	2.10
LPb	38.1	46.0	0	21 700	262 000	11.9
LPb40	—	—	—	2800	6400	2.26

LPa and LPb, initial materials; LPa40 and LPb40, after 40 days of thermal oxidation; LPa80, after 80 days of thermal oxidation.

as a filler in addition to commercial Addiflex HE (5% w/w) pro-oxidant additives based, according to the producer, on manganese ions.

The second material (LPb) was low density polyethylene transparent film, 30–35 µm thick containing pro-oxidant additives based on Mn and Fe stearates. To compare both materials transition metal contents and molecular weight distribution parameters of initial and oxidized films were determined and are summarized in Table 1.

#### 2.2. Abiotic thermal oxidation, FTIR, GPC, elongation at break, metal contents

Sample films were cut into strips 10 cm long and 2 cm wide and incubated at 70 °C in air. At predetermined times, the strips were taken out and their transmission FTIR spectra (Mattson 3000, UNICAM, UK) recorded. The carbonyl index was calculated as the ratio of absorbance values at 1713 and 1465 cm<sup>-1</sup>.

Information about molecular weight distribution was obtained by gel permeation chromatography on a PL-GPC 220 instrument (Polymer Laboratories) with refractometric and viscometric detection, which was equipped with 3x PL gel 10 µm MIXED-B columns and used 1,2,4-trichlorobenzene as a mobile phase. All operations were done at 160 °C. The corresponding data obtained were processed with Viscotek TriSEC software.

Elongation at break was measured in Tensometer 2000 (Alpha Technologies) at 100 mm per minute; results are averages from 5 subsamples for each time point.

Metal content was analyzed by ICP-MS (Agilent 7500 CE) equipped with collision cell microwave digestion (Milestone MLS1200) according to the manufacturers instructions.

#### 2.3. Biodegradation

Samples, previously oxidized in the aerated oven for 40 days (LPa40 and LPb40) and 80 days (LPb80), were disintegrated into fragments less than 2 mm, and mixed with mature compost (or fresh forest soil) and perlite in the ratio 1.5: 2.5: 10. All components were weighed in biometric flasks (1.5 g of sample fragments per flask) equipped with septa on stoppers and fitted with another two valves for aeration between measuring cycles. Sample flasks were incubated at 25 °C for biodegradation in the soil environment and at 58 °C for biodegradation under compost conditions. Head space gas was sampled at appropriate intervals through the septum with a gastight syringe and then injected manually into a GC instrument (Agilent 7890). Sampling was operatively adapted to actual CO<sub>2</sub> production and O<sub>2</sub> consumption. From the CO<sub>2</sub> concentration found, the percentage of mineralization with respect to the initial sample carbon content was calculated. Endogenous production of CO<sub>2</sub> by soil or compost in blank incubations was always subtracted to obtain values representing net sample mineralization. In parallel, oxygen concentration was

also monitored to provide a control mechanism so as to ensure samples did not suffer from hypoxia. Three parallel flasks were run for each sample, along with four blanks and positive control flasks with microcrystalline cellulose for both compost and soil.

#### 2.4. Microscopy

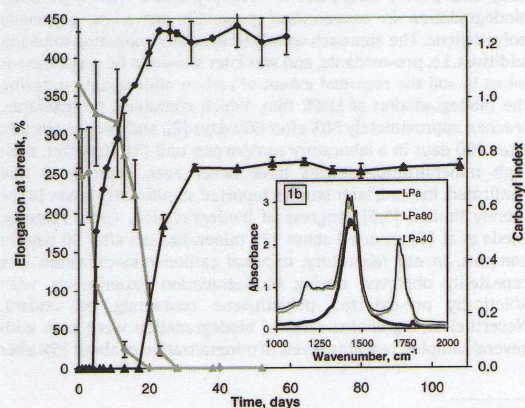
First, PE film fragments were fixed with 4% formaldehyde (overnight at 4 °C) and then stained with carbolfuchsin for optical microscopy or coated with thin gold/palladium layer for scanning electron microscopy on VEGA LMU (Tescan s.r.o., CZ).

### 3. Results and discussion

#### 3.1. Abiotic oxidation

The two investigated samples were first incubated at 70 °C in an aerated oven and the degree of oxidation monitored by FTIR (Fig. 1). This procedure was necessary to promote a certain level of pro-oxidant catalyzed oxidation in the samples, thereby simulating several years of ageing at an environmental temperature. As shown in Fig. 1, it seems that in cases of both samples the oxidation reached some extent over a period of time and then remained constant. Here the plateau appears on the curve after approximately 33 days and 23 days for LPa and LPb, respectively. Despite about one-third lower pro-oxidant content LPb displayed shorter induction period (about 5 days) and, in particular, significantly higher extent of oxidation at the end of the experiment. Actually the final carbonyl index of LPa was relatively low compared with similar materials and probably such behaviour could be attributed to some stabilization effect of calcium carbonate filler. Analogous curves were also obtained at 60 °C and 80 °C [8], as well as with other similar materials in our laboratory (data not published). As a complementary parameter, elongation at break was additionally measured at identical time points. Shortly after entering a fast oxidation period, at around day 20 for LPa, the materials totally lost all strength and became very brittle. For both samples the curves showing the deterioration of mechanical properties were exactly opposite to those of material oxidation (Fig. 1).

In order to resolve the question of whether changes in material continued after the oxidation had reached the plateau and if these



**Fig. 1.** Carbonyl index (grey) and elongation at break (black) evolution during thermal oxidation of LPa (▲) and LPb (◆) at 70 °C. FTIR spectra of the LPa at selected time points in the inner section. Error bars corresponds to double a standard deviation ( $n = 3$  for carbonyl index and  $n = 5$  for elongation at break).



affected the biodegradability of the material, samples were prepared from LPa, which were aged for 40 (LPa40) and 80 (LPa80) days at 70 °C. Both samples had almost identical carbonyl indexes and spectra (Fig. 1b).

It is generally believed that molecular weight reduction during abiotic oxidation is of crucial importance to biodegradation [9]. Contrary to FTIR, molecular weight distribution curves revealed some evolution between LPa40 and LPa80 samples (Fig. 2). LPa80 distribution was significantly narrower, lacking some higher molecular weight components present in LPa40. The overall parameters of distributions are summarized in Table 1. Mw of LPa40 is strongly influenced by the long right shoulder adjacent to the major peak. Although some changes in distribution curves were observed, they were not dramatic. The part of the material around and below the molecular weight of 1000, believed to be the most susceptible to biodegradation, did not seem to change remarkably, but here it must be noted that there are limitations to the applied method's performance at such a low molecular mass range. The conclusion can be made that thermal oxidation changes in the material were significantly retarded after day 33. A lack of high molecular weight shoulder in LPa80 distribution can also be explained by subsequent processes, e.g. cleavage of ester bonds [10] or disruption of some intermolecular bridges formed during thermal oxidation.

LPb was analyzed after 40 days of pre-oxidation at 70 °C. In agreement with higher carbonyl index attained molecular weight distribution was shifted even more towards lower values than in case of LPb. On the other hand molecular weight distributions of both initial materials were comparable (Fig. 2, Table 1).

LPb40 was completely soluble in 1,2,4-trichlorobenzene at 160 °C, insoluble portions of LPa40 and LPa80 were equivalent to the filler content, so the samples contained no or only a little gel.

### 3.2. Biodegradation in soil and compost environments

Pre-oxidized LPa40, LPa80 and LPb40 samples were tested in laboratory biodegradation experiments. The aim was to evaluate biodegradation of the materials in a soil environment and in compost, to find out whether there are differences in the extent and/or course of biodegradation between LPa40, LPa80 samples and to compare the sample containing calcium carbonate filler (LPa40) with similar sample without the filler (LPb40).

In soil (Fig. 3), during the first few days, significant production of carbon dioxide was observed mainly for LPa40, LPa80, marking rapid consumption of easily degradable small molecular weight

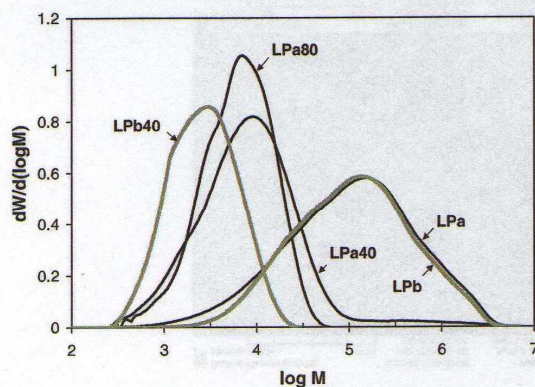


Fig. 2. Changes in molecular weight distributions during thermal oxidation at 70 °C.

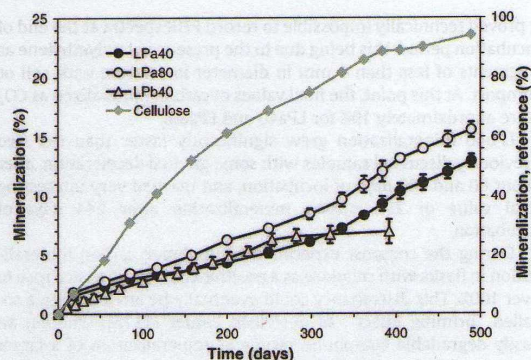


Fig. 3. Biodegradation in soil environment. Carbon mineralization of the reference compound (cellulose) is displayed on the right axis. Error bars corresponds to double a standard deviation ( $n = 3$ ).

compounds which are released from the material [5]. Then all samples entered into a period of slower  $\text{CO}_2$  production, with LPa40 possessing a slightly higher slope with respect to LPa80, which could be attributed to greater abundance of biodegradable material in the longer thermally-oxidized sample. From around day 300, LPa40, LPa80 curves progressed approximately in parallel, and their slopes remained significantly positive till the end of the observation period, thus witnessing continuous biodegradation in both samples. After more than 460 days, the final values of carbon mineralized as  $\text{CO}_2$  were about 13% in the case of LPa40 and about 16% for LPa80. In contrast with previously documented higher oxidation and more important fragmentation of polymer chains in LPb,  $\text{CO}_2$  release from this sample nearly stopped after 280 day of incubation and final values of about 7% mineralization were significantly lower than that for both LPa40 and LPa80.

In compost at 58 °C (Fig. 4), rapid biodegradation again took place initially for all samples.  $\text{CO}_2$  production in LPa40 and LPa80 samples was identical. This means that the higher temperature in compost incubation promoted changes in material that relatively quickly equalized the susceptibility of both samples towards biodegradation. It might also be suggested that the higher temperature probably did not initiate further alterations in a longer oxidized sample, as LPa80 did not maintain its lead. From approximately day 200, the biodegradation rate slowed down a fraction.

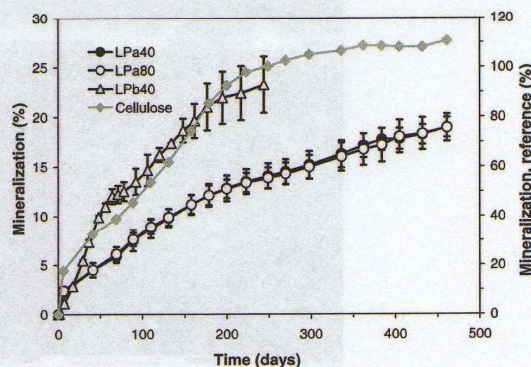


Fig. 4. Biodegradation in compost environment. Carbon mineralization of the reference compound (cellulose) is on the right axis. Error bars corresponds to double a standard deviation ( $n = 3$ ).



It proved technically impossible to record FTIR spectra at the end of incubation period, this being due to the presence of polyethylene as fragments of less than 1 mm in diameter in mixture with soil or compost. At this point, the final values of carbon mineralized as  $\text{CO}_2$  were approximately 19% for LPa40 and LPa80.

LPb40 mineralization grew significantly faster than the two previously discussed samples with some gradual deceleration, after about 60 and 180 days of incubation, and reached very interesting final value of 23% carbon mineralization after 244 days of incubation.

During the compost experiment, cumulative carbon mineralization in flasks with cellulose as a positive control substance rose to over 100%. This discrepancy could eventually be attributed to a so-called “priming effect”, as described earlier [11,15], wherein an easily degradable compound promotes mineralization of a larger amount of carbon from compost matter than occurs in a blank experiment.

LPa contained 15% (w/w) of micro-milled calcium carbonate filler. In order to evaluate the amount of carbon mineralized as  $\text{CO}_2$

during biodegradation, it is necessary to take into account that some of the  $\text{CO}_2$  evolved can actually originate from the filler. Considering the known composition of the material, carbon from  $\text{CaCO}_3$  accounts for about 2.5% of the total carbon in the material. Therefore, should all the carbon in  $\text{CaCO}_3$  be released as  $\text{CO}_2$ , 2.5% would have to be subtracted from the final mineralization results listed above. We believe that the real percentage of recorded  $\text{CO}_2$  originating from the filler was significantly lower.

### 3.3. Microscopic observation

At the end of the incubation period, some of the material fragments were withdrawn from the incubation flasks and inspected via optical and electron scanning microscopy. In addition, electron microscopy of the initial material and the samples following thermal oxidation was conducted. In both microscopy techniques there was no marked difference between the LPa40 and LPa80 samples, although some differences between compost and soil could be seen (Fig. 5). The surface of the initial LPa material when

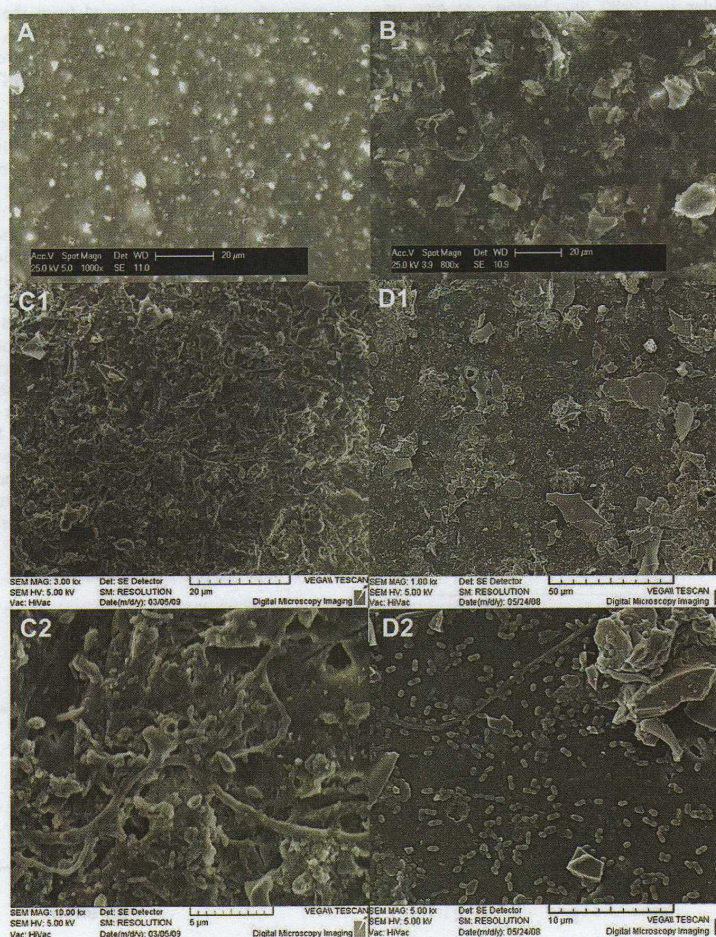


Fig. 5. Scanning electron microscopy of LPa surface. A, initial material (mag. 1000 $\times$ ); B, after 40 days of thermal oxidation at 70 °C (mag. 800 $\times$ ); C1, after about 450 days in compost (mag. 3000 $\times$ ); C2 detail of C1 (mag. 10 000 $\times$ ); D1, after about 450 days in soil (mag. 1000 $\times$ ); D2 detail of D1 (mag. 5000 $\times$ ).



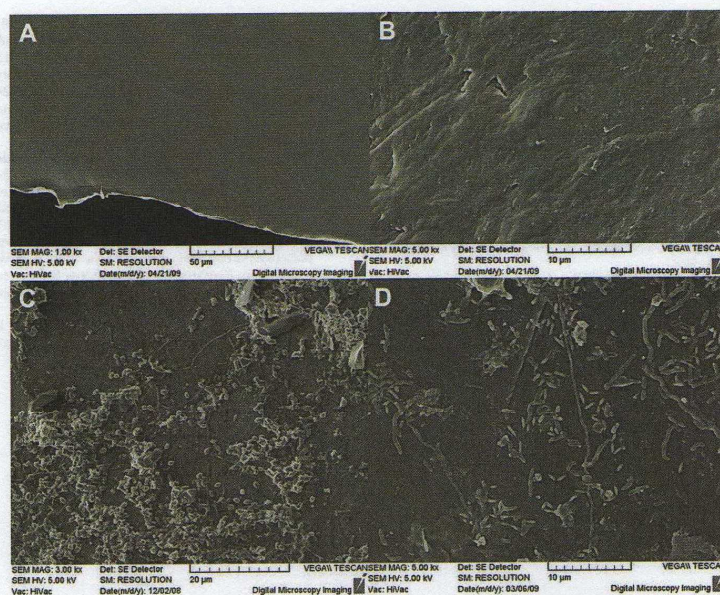


Fig. 6. Scanning electron microscopy of LPb surface. A, initial material (mag. 1000 $\times$ ); B, after 40 days of thermal oxidation at 70 °C (mag. 5000 $\times$ ); C, after about 200 days in compost (mag. 3000 $\times$ ); D, after about 350 days in soil (mag. 5000 $\times$ ).

intact was relatively smooth and grains of inorganic filler were visible as highly reflexive white spots using the material contrast enhancing WD detector (Fig. 5a). After thermal oxidation, the appearance of the LPA material changed dramatically. The surface became highly deteriorated, with numerous chips of the material detaching completely from the surface (Fig. 5b). The appearance was also completely different from the material without filler (LPb40) or our experiences from previous studies [5], where only minor surface deterioration could be found on pre-oxidized samples after a thorough inspection at a higher magnification (Fig. 6b).

After biodegradation in compost and soil environments, the surfaces of all samples showed a relatively dense covering of microorganisms. In soil (Figs. 5d1, d2 and 6d), individual cells were visible and some segmented bacterial filaments were discovered in case of both LPA and LPb (Fig. 6d). Such features are typical for *rhodococci*, earlier suggested as playing a role in polyethylene biodegradation [12,13], although such identification is highly speculative. Optical microscopy (not shown) also revealed a high number of fungi filaments laying on the material's surface. Fungi filaments were not preserved and probably detached during preparation of the samples for electron microscopy. In compost, a dense network of non-segmented bacterial filaments formed, which were of a type characteristic for thermophilic *actinobacteria*, in addition to various individual bacterial cells (Figs. 5c1, c2 and 6c). Enospores are visible at the side branches of filaments in the figure with higher resolution (Figs. 5c2 and 6c). Overall, it could be expressed that the relatively dense microbial colonization was in accordance with the significant degree of biodegradation determined. Moreover, it is our suggestion that the presence of filler led to greater deterioration of the surface, which consequently increased the availability of biodegradable compounds and thereby biodegradation of the material especially in soil incubation experiments.

#### 4. Conclusions

During the observation period all pre-oxidized materials investigated were significantly biodegraded in both soil and compost environments. The interesting point of the study appears to be the discrepancy when comparing the relative results of samples containing the filler (LPA40 and LPA80) and the sample without filler (LPb40) in soil and compost. It seems, that the presence of calcium carbonate filler was likely to have positively influenced the bioavailability of biodegradable compounds in the material and observed carbon mineralization. In particular, the extent of biodegradation in soil was important and higher with respect to the material without filler. Earlier, similar biodegradation promoting effect was observed with montmorillonite filled oxo-biodegradable polyethylene [14]. On the other hand the filler has negative effect on the abiotic pre-oxidation of the sample. The observation, that more oxidized but less deteriorated sample LPb exhibited faster mineralization in the compost experiment, could be explained by the idea that there the diffusion and bioavailability of the biodegradable fraction of the material was supported by higher temperature so the higher total content of this fraction prevailed. Differences between microflora in compost and soil could also be some part of the explanation.

At the end of the incubation period, slopes of biodegradation curves were still significantly positive (except of LPb40 in soil) thus highlighting ongoing biodegradation. Comparing the LPA40 and LPA80 samples revealed that even when the carbonyl index reached its plateau, some changes in the material can continue and influence the biodegradability of the material.

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## Příloha P 3: Biodegradace PVA za denitrifikačních podmínek

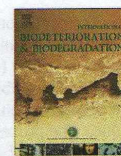
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### Polyvinyl alcohol biodegradation under denitrifying conditions



Hana Marušincová<sup>a</sup>, Lucie Husárová<sup>a,b</sup>, Jan Růžicka<sup>a,b</sup>, Marek Ingr<sup>c,d</sup>, Václav Navrátil<sup>d,e</sup>,  
Leona Buňková<sup>a</sup>, Marek Koutny<sup>a,b,\*</sup>

<sup>a</sup>Tomas Bata University in Zlín, Faculty of Technology, Department of Environmental Protection Engineering, nám. T.G.Masaryka 5555, 760 01 Zlín, Czech Republic

<sup>b</sup>Tomas Bata University in Zlín, Centre of Polymer Systems, nám. T.G.Masaryka 5555, 760 01 Zlín, Czech Republic

<sup>c</sup>Tomas Bata University in Zlín, Faculty of Technology, Department of Physics and Material Engineering, nám. T.G.Masaryka 5555, 760 01 Zlín, Czech Republic

<sup>d</sup>Charles University in Prague, Department of Biochemistry, Hlavova 2030, 128 43 Prague 2, Czech Republic

<sup>e</sup>Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo Sq. 2, 16610 Praha 6, Czech Republic

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#### ABSTRACT

Polyvinyl alcohol was biodegraded under denitrifying conditions with a microbial community originated from a municipal wastewater treatment plant. The derived microbial consortium was capable of polyvinyl alcohol degradation under both denitrifying and aerobic conditions. The community dynamics was monitored by temperature gradient gel electrophoresis, and a principal utilizing organism was identified and assigned as *Steroidobacter* sp. PD. The possible role of *Steroidobacter* sp. PD was also investigated by sequencing the 16S rDNA clone library prepared from the degrading community. qPCR analysis showed that the fraction of the microorganism in the community was very low initially (0.02%) and had reached to about 16% by the end of the biodegradation experiment. The study revealed that polyvinyl alcohol can be biodegraded in a water environment not only under aerobic but also under denitrifying conditions.

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#### 1. Introduction

Polyvinyl alcohol (PVA) is a water soluble polymer produced on a mass scale. Its annual production exceeds 1 megaton and a steady rise in this figure (IHS Handbook, 2007) is discernible. PVA is used as a thickening, emulsifying or film-forming agent or as an adhesive in many household and industrial applications, especially in the paper, textile and chemical industries. Due to such mass production and utilization a considerable amount is expected to leak from the afore-mentioned processes into the environment, especially into waste-water.

Fortunately, PVA was found to be biodegradable despite its full carbon backbone. It has, however, also gradually become apparent that PVA-degradation capacity is not ubiquitous, and that it is rather sparsely distributed among some bacterial and fungal taxa. Most such bacterial degraders were classified among pseudomonads but especially sphingomonads (Kawai, 1999). Some other bacterial

degraders comprise Gram-negative species like *Alcaligenes faecalis* (Matsumura et al., 1994) but also Gram-positive ones, e.g. *Bacillus megaterium* (Mori et al., 1996). Examples of some fungal degraders identified so far include *Penicillium* (Qian et al., 2004), and *Aspergillus* (Jecu et al., 2010; Stoica-Guzun et al., 2011). From the literature it is obvious that substantial biodegradation can mainly be expected in an aerobic aquatic environment, while PVA removal from soils is very limited and can be mostly a result of the non-specific action of lignolytic enzymes (Mejia et al., 1999; Chiellini et al., 2003). There are some indications describing partial anaerobic removal of relatively low molecular weight PVA (Matsumura et al., 1993; Gartsier et al., 1998) or partial anaerobic removal of PVA as a component of a polymer blend with other easily biodegradable constituents (Hrnčířik et al., 2010). Yu et al. (Yu et al., 1996) have suggested that the addition of nitrates could enhance PVA removal in a sequential anaerobic–aerobic bioreactor. However, none of these reports identified the relevant microorganisms or judged at least whether a specific PVA degradation was observed.

Two principal enzyme systems have been described. The first one, which relies on secondary alcohol oxidase (Shimao et al., 1983) with oxygen as an electron acceptor, can in principle be functional under

\* Corresponding author. Tomas Bata University in Zlín, Faculty of Technology, Department of Environmental Protection Engineering, nám. T.G.Masaryka 5555, 760 01 Zlín, Czech Republic. Tel.: +420 576031409.  
E-mail address: [mkoutny@ft.utb.cz](mailto:mkoutny@ft.utb.cz) (M. Koutny).



aerobic conditions only. The second, constitutes a PVA degradation pathway beginning with the action of PQQ containing periplasmic dehydrogenase (PVADH), from which electrons are probably conducted to soluble cytochrome *c* and further toward a terminal acceptor; oxygen, most likely under aerobic conditions (Shimao et al., 1996). The process should be feasible under anaerobic conditions in the presence of an alternative electron acceptor, e.g. nitrogen containing electron acceptors (Zumft, 1997), Reimann et al., 2007). Subsequently,  $\beta$ -hydroxyl ketone products of PVA dehydrogenase can be hydrolyzed in an aldolase like reaction catalyzed by a related hydrolase (Hirota-Mamoto et al., 2006; Kawai and Xiaoping, 2009), and shorter fragments of the polymer can then probably be assimilated.

The authors sought to investigate such a possibility in a series of experiments. The study reports on PVA specific biodegradation under denitrifying conditions and brings to light evidences of PVA consumption related to denitrification, in addition to identifying microorganisms relevant to the process.

## 2. Materials and methods

### 2.1. Sampling of the microbial community

Waste-water sludge from the denitrifying compartment of the Zlín-Malenovice municipal waste-water treatment plant (Czech Republic) was sampled in March 2010, and then on two more occasions at about two-month intervals. The sludge was immediately purged with nitrogen, filtered through a screen (4 mm<sup>2</sup>) to remove macroscopic particles, washed twice with mineral medium of pH 7.2 (Muchova et al., 2009) by centrifugation (5000 g, 12 min), and purged with nitrogen again.

### 2.2. Biodegradation experiments

Incubations were conducted in glass bottles (total volume, 320 ml) containing 280 ml of the mineral medium (in g l<sup>-1</sup>: KH<sub>2</sub>PO<sub>4</sub> 0.09, Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O 0.96, NH<sub>4</sub>Cl 1.0, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.1, Fe(NH<sub>4</sub>)<sub>2</sub>(-SO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O 0.03, CaCl<sub>2</sub> 0.01, KNO<sub>3</sub> 0.86, trace element solution (Muchova et al., 2009) 1.0 ml), 100 mg l<sup>-1</sup> of PVA (POVAL 205, 87–89% of hydrolysis; Kuraray Co. Ltd., Japan), 500 mg l<sup>-1</sup> of NO<sub>3</sub><sup>-</sup> (as KNO<sub>3</sub>), 1 g l<sup>-1</sup> dry weight of the preprocessed sludge from the first instance of sludge sampling. The cultures were purged with nitrogen and sealed with stoppers equipped with a rubber septa and incubated anaerobically on a magnetic stirrer (each bottle contained a magnetic bar; 250 rpm) at 25 °C. The experiments were carried out in three replicates. As an alternative, the PVA biodegradation experiment was also conducted under an aerobic condition in 500 ml flasks equipped with gas permeable stoppers filled with 100 ml of identical media under vigorous shaking (180 RPM).

The PVA concentrations were assayed in microplates (Joshi et al., 1979; Vaclavkova et al., 2007). After removing biomass by centrifugation (15,000 × g, 15 min), 20  $\mu$ l of a sample, 42  $\mu$ l of boric acid solution (40 g per liter) and 10  $\mu$ l of I<sub>2</sub>/KI solution (12.7 g I<sub>2</sub> and 40 g KI per liter) were pipetted into a well, and after 5 min an absorbance reading of 660 nm was made. Actual PVA concentration was deduced from a calibration curve.

Nitrates were assayed potentiometrically with an ion-selective electrode (Perfection NO<sub>3</sub> combination, Mettler Toledo) according to the manufacturer's instructions. KNO<sub>3</sub> solutions in above described mineral medium were used for calibration.

### 2.3. Isolation procedures

#### 2.3.1. Preparation of enriched PVA degrading consortia

Fresh media of the described composition were inoculated with preprocessed sludge from the second instance of sludge sampling

and cultivated for 60 days under identical conditions. PVA consumption was verified and the culture was used to inoculate another fresh medium, and this cultivation was held for 30 days. Then the microorganisms were collected by centrifugation (5000 × g, 12 min) and used for inoculating fresh medium with increased concentrations of nutrients (PVA, 150 mg l<sup>-1</sup>; NO<sub>3</sub><sup>-</sup>, 1000 mg l<sup>-1</sup>); the initial dry weight of the inoculum was set to 100 mg l<sup>-1</sup>. After four weeks of cultivation the culture was diluted ten times with fresh medium of the latter composition and cultivated again for another four weeks. Following this, the culture was supplemented three times in three week intervals in a manner that the PVA concentrations were set to 200 mg l<sup>-1</sup> and nitrate concentrations to 1000 mg l<sup>-1</sup>; this culture was designated "B". In an identical way the sludge sample from the third instance of sampling was processed and the final culture designated "D". After every manipulation, cultures were always purged with nitrogen and sealed.

#### 2.3.2. Isolation attempts

After the enrichment procedure, microorganisms from "Culture B" were collected by centrifugation, carefully resuspended, and a series of dilutions were prepared and transferred onto agar plates containing mineral agar with nitrate (BMA, control), mineral agar with nitrate and 500 mg l<sup>-1</sup> PVA (BMA-PVA), and mineral agar with nitrate, succinate and ethanol (BMA-SE, 500 mg l<sup>-1</sup> of each carbon source). Variants of the described solid media containing 20  $\mu$ g l<sup>-1</sup> of pyrroloquinolino quinone (PQQ) were also used. The plates were incubated at 25 °C anaerobically (anaerostat MERCK, Anaerocult A) or under aerobic condition.

#### 2.4. DNA isolation, PCR and TGGE conditions

DNA from denitrifying cultures was extracted using a commercial DNA extraction kit (PowerSoil, MoBio) according to the manufacturer's instructions, involving an initial bead-beating step. The primers fd1 and rD1 (AGAGT TTGAT CCTGG CTCAG and AAGGA GGTGA TCCAG CC, respectively) were used to amplify nearly a full-length 16S rRNA gene (Weisburg et al., 1991). Each 25  $\mu$ l PCR reaction contained 12.5  $\mu$ l of GoTaq Green hot start master mix (Promega), 1  $\mu$ l of each primer solution (12.5 pmol), 9.5  $\mu$ l of water for molecular biology, and 1  $\mu$ l (5–10 ng) of bacterial DNA. All amplifications were carried out in Piko Thermal Cycler (Finnzymes); the temperature profile was as follows: initial denaturation at 94 °C for 5 min; 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. Subsequently, 1  $\mu$ l of the first PCR product was used as a template for nested PCR amplifying the V3-hypervariable region of the 16S rRNA gene with the primer pair 341fGC and 518r (ATTAC CGCGG CTGCT GG and CCTAC GGGAG GCAGC AG, respectively), where the GC clamp (CGCCC GCCGC GCGCG GCGGG CGGGG GCGGG GCAGG GGGGG) was covalently attached to the 5' end of the forward primer (Muyzer et al., 1993). The temperature program consisted of 1 min at 94 °C and 30 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, and a final extension at 72 °C for 10 min. TGGE separation of the amplified PCR products was achieved on a TGGE Maxi system (Whatman-Biometra) in an isocratic denaturing gel (8% acrylamide, 20% deionized formamide, 1× TAE, 2% glycerol and 8 M urea). Two to five micro liters of PCR products (60–100 ng DNA) were loaded into each well. A 100-bp DNA ladder (NEB) was loaded as a marker (Das et al., 2007). Electrophoresis was performed at the constant voltage of 130 V for 18 h and the optimal gradient was found to be from 35 °C to 55 °C. Afterward, the electrophoresis gels were stained with GelStar (Cambrex), according to the manufacturer's instructions, and documented. The whole procedure was repeated twice with identical results.



### 2.5. Phylogenetic analysis

Phylogenetic analysis of relationships between sequences retrieved from TGGE bands and the most closely related GenBank sequences were conducted in MEGA 5.05 (Nei and Kumar, 2000; Tamura et al., 2011). The distance was computed using the Neighbor-joining p-distance method and bootstrap test (1000 replicates).

### 2.6. Clone library and sequencing

PCR fragments containing almost full-length 16S rRNA genes from the investigated samples were amplified with the help of fD1, rD1 primers equipped with cohesive adaptors (ATGGG ATCCA GAGTTTGATC CTGGC TCAAG, CAGCT GCAGA AGGAG GTGAT CCAGC C) under the above-described PCR conditions, purified and subsequently ligated into pUC19 vector between the restriction sites EcoRI and HindIII with the aid of an In-Fusion HD Cloning Kit (ClonTech) following the manufacturer's instructions. Then CaCl<sub>2</sub>-competent *Escherichia coli* DH5 $\alpha$  cells were transformed with the ligation mixture, and transformants were selected on ampicillin containing LB (Sigma–Aldrich) agar plates. About 60 clones were selected randomly, and plasmid DNAs were isolated via a High-Speed Plasmid Mini Kit (GeneAid). The clones were checked for correctness of the ligation by restriction analysis using EcoRI and HindIII restriction endonucleases. The sequences of correct-length inserts were determined (JCU, Czech Republic).

### 2.7. Quantification of the PVA degrading strain by qPCR

A quantitative polymerase chain reaction (qPCR) was used in order to determine the relative amount of the PVA-degrading strain in the bacterial culture. The total number of bacteria in the culture was determined using the primers 341f and 518r described in (Muyzer et al., 1993), which anneal to the conserved sequences spanning the V3 hypervariable region of 16S rRNA gene and should, therefore, amplify the genomic sequence of any bacterial strain. Another primer pair was used to quantitate the PVA-degrading strain, which was designed to selectively amplify the 16S rRNA of the degrader with respect to other 16S rRNA sequences obtained by the cloning method described above. More specifically, these primers amplify a 220 bps portion of the V5 and V6 hypervariable regions of the gene (sequences: AGGGT CTGCC TCTCG GTG and CGCAT CTCTG CAGGA TTCC). All PCR reactions were carried out in triplicate in LightCycler 96-well plates sealed with adhesive foil (Roche) using a LightCycler 480 II instrument (Roche) in a total volume of 10  $\mu$ l. Each reaction consisted of 5  $\mu$ l of LightCycler 480 SYBR I Green Master (Roche), 1  $\mu$ l of template DNA (isolated DNA from bacterial culture or plasmid standard) and 2  $\mu$ M of final concentrations of both forward and reverse primers. In order to eliminate contamination by ambient bacteria, which might cause a false positive signal when using universal bacterial primers, stock solutions of primers, water and the 96-well plate were irradiated with UVC light ( $\lambda = 254$  nm) for 30 min prior to mixing the reaction. Initial denaturation for 1 min at 95  $^{\circ}$ C was followed by 45 cycles of 10 s at 95  $^{\circ}$ C, 30 s at 56  $^{\circ}$ C, and 30 s at 72  $^{\circ}$ C. The threshold cycle numbers were then determined from fluorescence intensities acquired during the PCR runs by the 2nd derivative maximum method using LightCycler 480 software (Roche).

Serial tenfold dilution of the 16S rRNA gene of the degrader cloned in pUC19 plasmid was in parallel amplified with both primer pairs and served thus as standard for absolute quantification of both targets. Initial concentration of plasmid DNA prior to dilution was determined spectrophotometrically at 260 nm (Nanodrop ND-1000, Thermo Scientific). The concentration ranges of these

standards covered such ranges actually measured in the samples. The relative amount of the degrader was then calculated as the ratio of the 16S rRNA gene copy number, as determined by amplification with specific-primers, to the 16S rRNA gene copy number determined by amplification with universal primers.

### 2.8. DNA sequences

Sequences from the clone library were deposited in a GenBank, the accession numbers running from JQ726646–JQ726696. Almost a full 16S rDNA sequence of *Steroidobacter* sp. PD possesses the GenBank accession number JQ726645.

## 3. Results

### 3.1. PVA biodegradation experiment

Minimal mineral medium with nitrate content was supplemented with PVA as the sole carbon substrate, and inoculated with sludge from the denitrifying compartment of the municipal wastewater treatment plant. These cultures were flushed with nitrogen, sealed and incubated anaerobically. At regular intervals samples were taken and the PVA concentration monitored. Initially, the polymer concentration remained stable, until about day 12 when the apparent lag-phase seemed to be over and a relatively fast decrease in PVA concentration commenced (Fig. 1). This experiment was repeated several times with the same sludge sample and the sludge samples drawn from the same location, but about two months later and then again four months later, with very similar results. If the same medium was inoculated with the microbial community sampled at the end of the previously described experiment, in other words with the PVA-acclimated microbial community, no lag-phase was observed and PVA was quickly consumed (Fig. 1). Nitrate concentration decreased along with PVA biodegradation and, on the contrary, PVA concentration remained almost unchanged if no nitrate was present in the medium (Fig. 1), so PVA degradation in the absence of oxygen was apparently dependent on the presence of nitrate. These observations could indicate that the original sludge contained a relatively low concentration of a microorganism or a microbial consortium able to

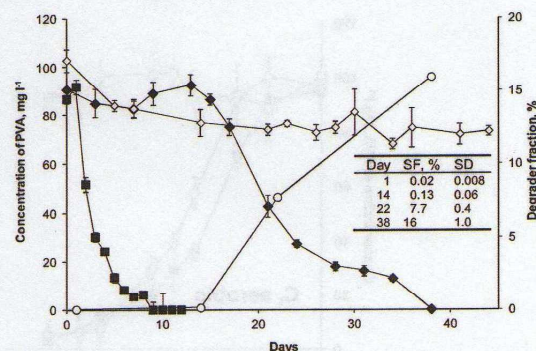


Fig. 1. PVA concentration in biodegradation experiments under various conditions.  $\blacklozenge$ , mineral medium inoculated with a PVA-non-adapted sludge microbial community with 500 mg L<sup>-1</sup> nitrate;  $\blacksquare$ , mineral medium inoculated with a PVA-adapted sludge microbial community with 500 mg L<sup>-1</sup> nitrate;  $\diamond$ , mineral medium inoculated with a PVA-non-adapted microbial community without nitrate;  $\circ$ , *Steroidobacter* fraction in the bacterial community. The table inserted shows degrader fractions (SF) at respective points in time. Values are given as means  $\pm$  1 standard deviation ( $n = 3$ ).



degrade and assimilate PVA under conditions supporting denitrification.

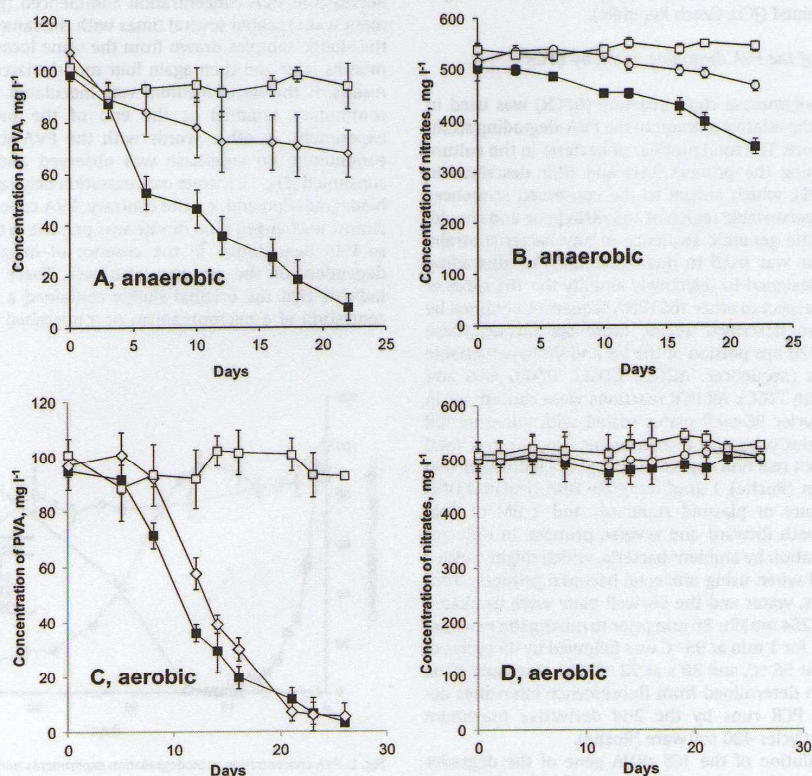
Another series of experiments was conducted with the enriched PVA degrading consortium "D" obtained by the procedure described. Again, under anaerobic conditions, PVA was consumed in the presence of nitrates whereas in their absence only a slight decrease in PVA concentrations at the beginning of the observation period was observed (Fig. 2A). Simultaneously, nitrate concentrations were monitored (Fig. 2B), showing that PVA degradation was accompanied by said nitrate consumption. About 150 mg of the nitrates per liter was consumed, which corresponds to about 60% of the theoretical amount necessary for the complete mineralization of the PVA inserted (about 240 mg of nitrates per 100 mg of PVA). Some degree of nitrate consumption in the incubation without PVA can be explained by the denitrification of carbon substances introduced with the sludge biomass (flocs) used as the inoculum.

An identical series of experiments was repeated under aerobic conditions. Here (Fig. 2C), both cultures, with and without nitrates, exhibited a rapid decrease in PVA concentrations, witnessing the ability of the consortia to degrade the polymer aerobically and suggesting that the key organisms of the consortium probably are facultative anaerobes. No nitrate consumption was detected in any variant of the media (i.e. with or without PVA, Fig. 2D), which is well consistent with the general idea of aerobic-anaerobic

metabolisms switching and the preference for oxygen in facultative anaerobic denitrifiers (Zumft, 1997).

### 3.2. Isolation attempts

The active microbial communities from biodegradation experiments were subjected to the described series of dilutions and reinoculations with the aim to enrich the key microorganisms and to dilute out the others. Dilutions of the enriched culture designated "B" were transferred onto the described selective agar media. After two months of cultivation no differences were observed between control BMA and BMA-PVA plates, distinct colonies were, however, found on BMA-SE plates, and finally 21 pure strains were isolated. Subsequently these strains were individually tested in liquid media for PVA biodegradation under denitrifying conditions, with and without PQQ addition, and for denitrification with succinate and ethanol. Despite the majority of the strains being capable of denitrification with the standard organic substrates, neither of them was able of PVA biodegradation under denitrifying conditions. Consortia composed from all isolated strains or their combinations did not prove active, either. Later the series of isolation attempts were repeated, in addition to which aerobic conditions were tested as well, but again without a positive result. After that, several further attempts for degrader's isolation were



**Fig. 2.** Biodegradation experiment with the enriched microbial consortium "D" under anaerobic and aerobic conditions. Panel A (anaerobic) and panel C (aerobic), concentrations of PVA; ■, inoculated mineral medium with 500 mg L<sup>-1</sup> of nitrates; ◇, inoculated mineral medium without nitrates; □, inoculated mineral medium with 500 mg L<sup>-1</sup> of nitrates, after preparation the culture was autoclaved. Panel B (anaerobic) and panel D (aerobic), concentrations of nitrates; ■, inoculated mineral medium with 100 mg L<sup>-1</sup> of PVA; ○, inoculated mineral medium without PVA; □, inoculated mineral medium with 100 mg L<sup>-1</sup> of PVA; after preparation the culture was autoclaved. Values are given as means ± 1 standard deviation (n = 3).



made. Briefly, on the base of Fahrbach's description of *Steroidobacter* (Fahrbach et al., 2008), BMA plates amended with glutamate or acetate were inoculated with different dilutions of acclimated culture "B" and later the same was done with BMA plates with PVA amended with 5% of autoclaved acclimated culture "B" and BMA plates with partially oxidized PVA. Despite such effort and identification of many colonies found on above mentioned agars, none of them was identified as *Steroidobacter* or proved to be the degrader.

### 3.3. Investigation of PVA degradation by cultivation-independent techniques

Simultaneously, the PVA degradation experiment depicted in Fig. 1 was followed by temperature gradient gel electrophoresis (TGGE) based on amplification of the 16S rRNA gene V3 hypervariable region (Fig. 3). The microbial community in the experiment inoculated with the original sludge was sampled at time intervals, the DNA was isolated, and TGGE profiles compared with and examined against the PVA degradation curve. Time evolution of the

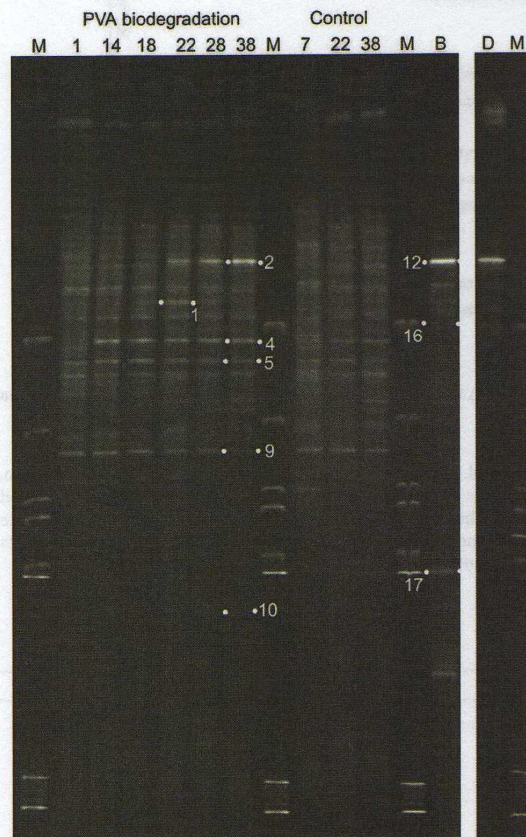


Fig. 3. TGGE profiles of the denitrifying PVA degrading microbial community. M, DNA marker; numbers on the top of the gel denote days during degradation or the control experiment (Fig. 1, ♦); B and D, microbial communities from enrichment cultures, where concentrations of the degraders were increased by the sequence of dilutions and transfers to fresh media. Sequences were successively obtained from the numbered bands and phylogenetic assignment made (Fig. 4).

TGGE profiles was also monitored in a blank experiment with an identical initial bacterial community but without PVA presence in the media.

It is quite obvious that a new band (Band 2) appeared between day 18 and day 22, which gradually increased in intensity. The same band was apparently missing in the control incubation. Comparing the time evolution of the investigated band with the curve in Fig. 1, one could see a clear concurrence of the onset of PVA consumption with the appearance of the band. Another interesting band (designated Band 1) exhibited a brief increase in intensity around day 22, which could also be related to the initiation of PVA consumption. The above-mentioned bands, along with other prominent bands from the last day of incubation, were excised and sequenced. The authors failed to obtain a quality sequence for some of them, which is often the case with TGGE. For the bands where sequences were successfully retrieved, their phylogenetic affiliations were sought out and are depicted in Fig. 4. Band 2 was assigned as a putative PVA utilizing organism closely related to the *Steroidobacter* strain found in the database.

The DNA from cultures used for isolation attempts, where enrichment for specific PVA degrading denitrifying microorganisms was induced, was processed in an identical way and the TGGE patterns visualized. Starting material for these incubations was sludge from the second and third instances of sampling at the same location. Both the obtained bacterial consortia "B" and "D" exhibited rapid consumption of PVA under denitrifying conditions, and both had highly intense bands at exactly the same position as the Band 2 previously identified. Later sequencing proved that these bands are identical with Band 2. It is worth mentioning that other significant bands found in patterns from the biodegradation experiment are apparently not present in patterns from enrichment cultures "B" and "D" and vice versa. These findings strongly supported the opinion that Band 2 represents a signal of a specific and dominant PVA utilizing organism in the described microbial community.

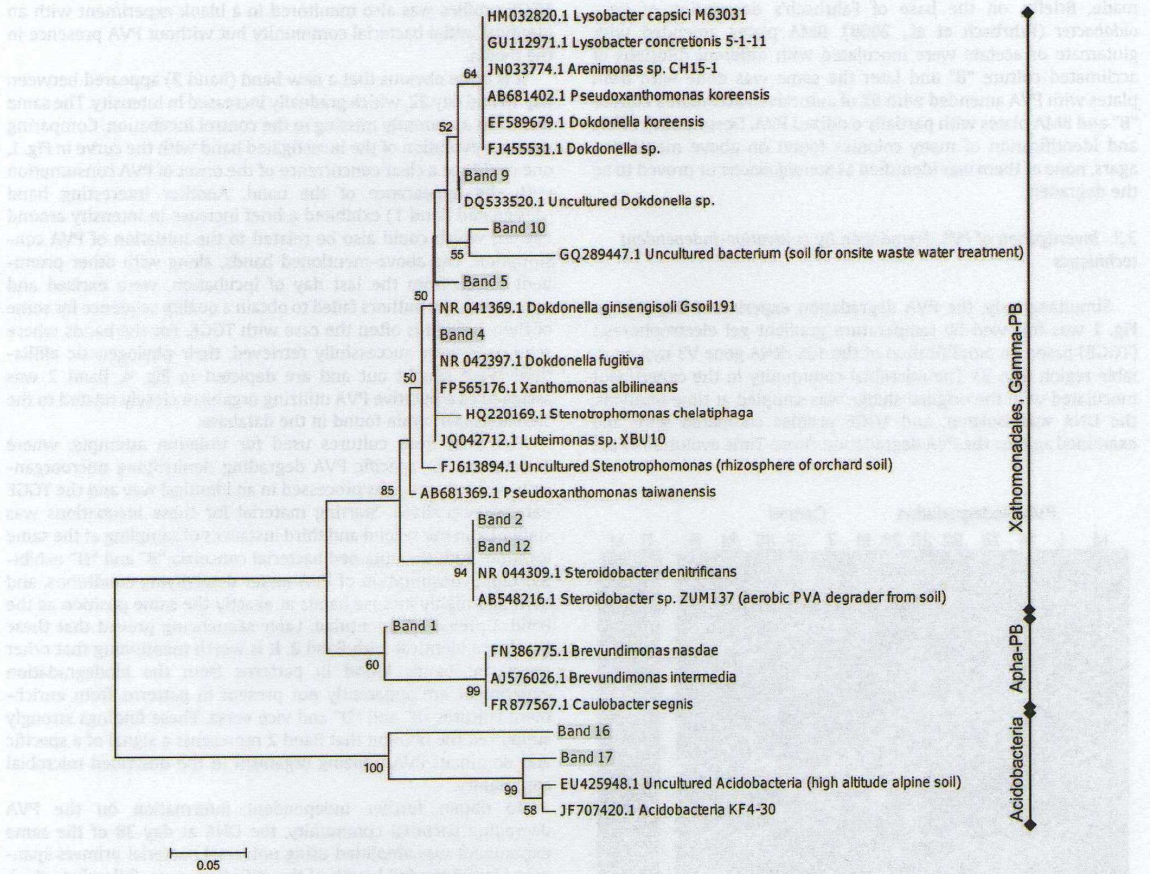
To obtain further independent information on the PVA degrading bacterial community, the DNA at day 38 of the same experiment was amplified using universal bacterial primers spanning almost the full length of the 16S rRNA gene, following which the fragments obtained were cloned and about 60 randomly selected correct clones were sequenced (Table 1). About 29% of the sequences from 51 correctly sequenced clones belonged to the bacterium previously identified from the TGGE band. Almost the complete length of its 16S rRNA gene was sequenced (GenBank JQ726645), the result revealing that the organism is closely related (97% identical) to the database strain *Steroidobacter* ZUMI 37 (GenBank AB548216.1); which, according to the note submitted with the sequence, was described as an aerobic PVA degrader isolated from soil.

Another dominant sequence found was closely related to the *Dokdonella* strain. Here assignment of the corresponding TGGE bands was not straightforward, as several relatively intense bands (Bands 4, 5, 9, and 10) could probably be related to this sequence. However, all these bands possessed constant intensity during the entire biodegradation experiment, and could also be found in the control incubation without PVA. Likewise, these bands are apparently missing or at least exhibit very low intensity in both enrichment cultures.

### 3.4. Monitoring the *Steroidobacter* fraction in the course of PVA biodegradation by qPCR

Absolute quantification of both *Steroidobacter* specific 16S rRNA and total 16S rRNA by qPCR was used to monitor the changes of the putative degrader fraction in the culture during the course of the





**Fig. 4.** Phylogenetic relationships of TGGE retrieved sequences and closely related sequences (GenBank). The distance was computed using the Neighbor-joining p-distance method and bootstrap test (1000 replicates). Only bootstrap values over 50% are shown. PB, proteobacteria.

PVA biodegradation experiment. DNA isolated from the bacterial culture samples taken on days 1, 14, 22, and 38 of the cultivation was used as template for qPCR reactions, each sample with both *Steroidobacter*-specific and universal primer pairs. The 16S rRNA gene of the organism cloned in pUC19 plasmid was used as a calibration

standard. Day 1 corresponded with the inoculation of the medium, while by day 38 almost all the PVA present in the medium had already been degraded. The fractions of *Steroidobacter* in the culture on the individual days of cultivation were calculated and the results were compared with the PVA degradation curve (Fig. 1).

**Table 1**  
Frequency and affiliations of retrieved sequences.

Clones <sup>a</sup>	Closest related organism, accession number	Identity	Taxonomic group
16/51	<i>Dokdonella</i> sp. LM 2–5, FJ455531.1	98%	Gamma-PB, <i>Xanthomonadales</i>
15/51	<i>Steroidobacter</i> sp. ZUMI 37, AB518216.1	97%	Gamma-PB, <i>Xanthomonadales</i>
7/51	Bacterium Ellin6095 ( <i>Burkholderia</i> ), AY234747.1	91%	Beta-PB, <i>Burkholderia</i>
2/51	<i>Caldilinea aerophila</i> DSM 14535, AB067647.1	86%	<i>Chloroflexi</i>
2/51	<i>Phenylobacterium koreense</i> MKC7, EF173338.1	99%	Alpha-PB, <i>Caulobacter</i>
2/51	<i>Candidatus Microthrix parvicella</i> M2, FJ638889.1	99%	<i>Actinobacteria</i>
1/51	Alpha proteobacterium ZLJ-0, GU247516.1	99%	Alfa-PB
1/51	<i>Chromatiaceae</i> bacterium MTPP2IF163, FN293071.1	98%	Gamma-PB
1/51	<i>Comamonadaceae</i> bacterium PIV-20-1, AJ505862.1	99%	Beta-PB, <i>Burkholderia</i>
1/51	<i>Paucibacter toxinivorans</i> 2B5, AY515391.1	89%	Beta-PB, <i>Burkholderia</i>
1/51	<i>Clostridium</i> sp. SH-C52, FJ424481.1	98%	<i>Firmicutes</i>
1/51	<i>Longilinea arvoryzae</i> , AB243673.1	84%	<i>Chloroflexi</i>
1/51	Alpha proteobacterium WD28, HQ341736.1	97%	Alfa-PB

<sup>a</sup> Number of given sequences versus total number of obtained sequences; PB, *Proteobacteria*.



This experiment shows that the PVA utilizing strain is present in the original sludge in a negligible fraction with respect to the other bacteria (~0.02%). During cultivation its fraction grew continuously up to the value of approximately 16%, which is in general accord with the result obtained from sequencing individual 16S rRNA clones amplified from the same bacterial culture. Moreover, the trend of the data corresponds with the degree of degradation of PVA in the culture, further supporting the findings obtained from the biodegradation experiment.

#### 4. Discussion

The biodegradation experiment proved that PVA degradation is possible under anoxic conditions using nitrate, as the terminal electron acceptor. These results indicated the presence of PVA degrading microorganisms that were probably initially present in a rather low concentration. To obtain further information, the microbiology of the process was investigated with both cultivation and cultivation-independent techniques. While attempts to isolate degrading strains on agar media, despite considerable effort, failed for the time being, TGGE revealed a distinct band which appeared to increase its intensity in correlation with decrease in PVA concentration. This band was sequenced, and later a corresponding almost full 16S rDNA sequence was obtained, showing high similarity with the 16S rDNA sequence of the previously identified aerobic PVA degrading strain *Steroidobacter* sp. ZUMI 37. The strain is also related to *Steroidobacter denitrificans* (GenBank EF605262.1), isolated as a denitrifying degrader of some steroid compounds (Fahrbach et al., 2008). This supports the idea that PVA utilization and denitrification could be connected to one organism. On the other hand, the inability to isolate the pure degrading strain on agar media could suggest that the PVA degrader requires another microbial partner(s) or some environmental factor(s) either for its growth or PVA utilization.

Other bands present in TGGE profiles seemed constant during incubation but also constantly present in the control incubation without PVA, therefore, they should not be connected with PVA biodegradation. The only other exception was Band 1, briefly increasing its intensity at the end of the lag phase. The sequence from the band was retrieved and found to be related to sequences from *Brevundimonas* strains. Interestingly, *Brevundimonas vesicularis* (formerly described as *Pseudomonas vesicularis*) was earlier identified as an aerobic PVA degrader (Watanabe et al., 1976). Although the band demonstrates low intensity in later phases of PVA degradation, sequencing the 16S rRNA gene library made at the end of the degradation period provided about 4% sequences which were affiliated with *Phenylobacterium koreense* MKC7 (Table 1), a member of the same taxonomic group *Caulobacteraceae* like *Brevundimonas*. Thus, it is possible that the organism represented by Band 1 could play a role at the beginning of degradation, while it is later overgrown by a *Steroidobacter* related strain (denoted further as *Steroidobacter* sp. DP), representing at the end about 29% of the bacterial community (according to the clone library sequencing).

Another suggestion about the role of *Steroidobacter* sp. DP comes from the TGGE patterns of cultures "B" and "D", originating from the second and third instances of sludge sampling, respectively, exhibiting both strongly dominant bands of the same organism. Microscopic observation of the enrichment cultures revealed the prevalence of Gram negative rods, which was consistent with the expected *Steroidobacter* phenotype. The relatively high number of other minor bands in the enrichment culture might result from the presence of the specified amount of acetate substituents on the PVA backbone that can be easily hydrolyzed and utilized by bacteria.

The correlation of PVA consumption and *Steroidobacter* sp. DP concentration was further confirmed by the qPCR experiment. Fractions of *Steroidobacter* sp. DP were calculated for several days selected during cultivation, and a clear trend was observed showing that this species grows in accord with PVA consumption. Moreover, the determined fraction at day 38 of the degradation experiment, which was 16%, agreed reasonably with the fraction of *Steroidobacter* sp. DP sequences retrieved from the clone library (29%). This small difference might be attributed to slightly different primer affinities in both methods, varying ligation efficiency for different sequences, the small set of sequenced clones, or a combination of these. Thus, a close correlation is observed between monitoring PVA consumption and growth of the *Steroidobacter* sp. DP fraction determined by qPCR, as well as between the final fraction of *Steroidobacter* sp. DP determined by sequencing clones and qPCR. All this evidence supports the hypothesis that PVA can be efficiently degraded during denitrification.

#### 5. Conclusion

In the described experiments polyvinyl alcohol was biodegraded under denitrifying conditions with a microbial community originated from a municipal waste-water treatment plant. *Steroidobacter* sp. DP was the principle PVA utilizing microorganism, and a number of indirect evidences suggest that the organism had an important role in PVA biodegradation under denitrifying conditions. Subsequent experiments also proved that the consortium dominated by *Steroidobacter* sp. DP is capable of aerobic PVA degradation. As stated in the introduction PVA is produced on a massive scale and in some cases could leak into waste-water or directly into the environment in considerable quantities. The findings presented here broaden our knowledge regarding the biodegradation of PVA and full carbon synthetic polymers in general. They also show that such processes can proceed in anaerobic environments as well, for example, in anaerobic compartments of waste-water treatment plants, but also in natural sediments, and raise the confidence in the relative environmental safety of PVA.

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## Příloha P 4: Vliv molekulové hmotnosti a formy PLA na její biodegradaci (Manuscript)

Lucie Husarova<sup>a,b</sup>, Silvie Pekařová<sup>b</sup>, Petr Stloukal<sup>a,b</sup>, Pavel Kucharzcyk<sup>a,b</sup>, Vincent Verney<sup>d,e</sup>, Sophie Commereuc<sup>c,d</sup>, Audrey Ramone<sup>d</sup>, Marek Koutny<sup>a,b,\*</sup>

<sup>a</sup>Tomas Bata University in Zlín, Centre of Polymer Systems, nám. T.G.Masaryka 5555, 760 01 Zlín, Czech Republic

<sup>b</sup>Tomas Bata University in Zlín, Faculty of Technology, Department of Environmental Protection Engineering, nám. T.G.Masaryka 5555, 760 01 Zlín, Czech Republic

<sup>c</sup>Clermont Université, ENSCCF, Institut de Chimie de Clermont-Ferrand, BP 10448, F-63000 Clermont-Ferrand

<sup>d</sup>Clermont Université, Université Blaise Pascal, Institut de Chimie de Clermont-Ferrand, BP 10448, F-63000 Clermont-Ferrand

<sup>e</sup>CNRS, UMR 6296, ICCF, BP 80026, F-63171 Aubiere

\*Correspondence author, email address: mkoutny@ft.utb.cz; phone: +420 604 559 681

### Abstract

Biodegradation of four poly(L-lactic acid) (PLA) samples with molecular weights (MW) in the interval from about 34 to about 160 kg mol<sup>-1</sup> was investigated under composting conditions. Biodegradation rate decreased and an initial retardation was discernible with increasing MW of the polymer. Also the specific surface area of the polymer sample was identified as the important factor accelerating biodegradation. Microbial community compositions and dynamics during the biodegradation of different PLA were monitored by temperature gradient gel electrophoresis, and were found to be virtually identical for all PLA materials and independent on MW. A specific PLA degrading bacteria was isolated and designated *Thermopolyspora flexuosa* FTPLA. The addition of limited amount of low MW PLA did not accelerated biodegradation of high MW PLA suggesting that the process is not limited with the number of specific degraders and/or the induction of specific enzymes. In parallel the abiotic hydrolysis was investigated for the same set of samples and their courses found quasi identical

with biodegradation of all four PLA samples investigated. This suggests that the abiotic hydrolysis represented a rate limiting step in the biodegradation process and the organisms present were not able to accelerate depolymerisation significantly by the action of their enzymes.

**Key words:** polylactic acid; biodegradation; abiotic hydrolysis.



## 1. Introduction

Plastic litter represents a serious environmental problem, provoking increasing concern among the broader public. The situation appears to be particularly alarming in developing countries without fully efficient waste management systems. Biodegradable polymeric materials represent a promising alternative to conventional polymers, at least for some applications [1,2]. Polylactid acid is a particularly promising member of this family of polymers [3,4]. Previously, applications of PLA were limited to the biomedical sector because of its high cost and relatively low molecular weight [5]. Recently, new techniques which allow for economical production of high molecular weight (MW) PLA (greater than 100,000 Da), with relatively good mechanical, thermal and processing properties, have brought about further diversity of PLA utilization [5,6]. Presently, this material can be used in a wide spectrum of products as a consequence, including packaging materials, mulching films and bottles, as well as in manufacturing fibers for nonwoven items, textiles and carpets [7]. PLA also represents a material that can be produced from renewable resources, as its monomer can be obtained by fermentation of various plant materials.

During its environmental degradation ester bonds of PLA must be cleaved either hydrolytically or by extracellular enzymes to enable the PLA monomers or oligomers to be assimilated. Both parallel processes can be influenced by several factors. The enzymatically catalyzed process depends on the presence of specific microorganisms, which seems to be sparsely distributed in some environments [1] and presumably by optical purity [8] and crystallinity of the material [9]. It was proposed that amorphous regions are more vulnerable towards hydrolysis by model enzymes [10]. Abiotic hydrolysis is

critically influenced by temperature [11], where its rates differ considerably in the interval between 20 and 60 °C. Crystallinity with the preference of amorphous regions seems to play a role as well [12].

In the compost environment PLA biodegradation proceeds relatively easily and was rather extensively documented [13,14]. Conditions, especially during the thermophilic phase of the composting process, appear favorable both in spite of the presence of degrading microorganisms [15] and the promotion of hydrolysis under elevated temperature [11].

It was suggested that abiotic hydrolysis represent a first step in PLA biodegradation but a clear data documenting such statement are not readily available [16]. On the other hand, experimental data proved very fast and efficient decomposition of PLA by some enzymes [15,17] and the presence of hydrolytic enzymes produced by PLA utilizing microorganisms [18,19]. Finally, the specific surface of the testing specimen can have an important role probably especially during first phases of the biodegradation process [20,21]. Quite recently Pantani and his colleagues [22,23] showed that D-monomer content and processing history had a critical influence on sample crystallinity which further influenced hydrolysis and biodegradability, where they also observed the importance of sample morphology.

As it was outlined, biodegradation of a particular PLA grade can be influenced by many conflicting factors. Here we attempted to test four available PLA grades processed each into three different sample forms under identical experimental conditions and monitored and evaluated parallel processes of hydrolysis and microbial decomposition by comparing data from different experimental techniques employed.

## 2. Materials and methods

### 2.1 Materials

Poly(lactic acid) samples PLA1 and PLA2, were synthesized through direct melt polycondensation of L-lactic acid [24,25]; PLA3 purchased from NatureWorks® Ingeo™ grade 2002D (USA), PLA4 from VUCHV (Slovak Republic).

### 2.2 Polymer processing

The original polymers were processed to obtain three other forms of polymer samples with different specific surface areas.

**Powder.** PLA solution in chloroform (12.5 mg.mL<sup>-1</sup>) was gradually dispensed into 2.5 volumes of ethanol and the mixture was vigorously stirred. Subsequently, the precipitate obtained was decanted and rinsed twice with ethanol to remove chloroform. Finally, the precipitated PLA was filtered out and dried on air for 24 hours to evaporate ethanol and the residues of chloroform. As a result, a fine powder with a high specific surface was obtained. Its inspection with scanning electron microscopy and subsequent image analysis (ImageJ) revealed porous particles of about 20 μm average size.

**Films.** PLA films 100 μm thick were compression molded via heating for 1 minute to the processing temperature of 180 °C, then molded for 2 minutes and then immediately cooled under pressure by transferring the material to a second press set to 25°C.

**Thin coating on an inert surface.** The exact amount of PLA (50 mg) was applied as a chloroform solution (5 mg mL<sup>-1</sup>) to the surface of a pre-weighed porous inert material (perlite, 5 g) in biometric flasks and stirred thoroughly. The solvent was then stripped out with air flow for 24 hours, leaving the polymer deposited as a

thin coating with a high specific surface area.

### 2.3 Gel permeation chromatography

Molecular weight and distributions were determined by gel permeation chromatography, carried out on the Breeze chromatographic system (Waters, Milford, MA) equipped with a PLgel Mixed-D column (300 × 7.8 mm, 5 μm; Polymer Laboratories, Ltd.) and detected with the Waters 2487 dual-absorbance detector at 239 nm. Data was processed via Waters Breeze GPC software (Waters), and the weight average molar mass (M<sub>w</sub>), number average molar mass (M<sub>n</sub>) and subsequently polydispersity indexes (M<sub>w</sub>/M<sub>n</sub>) were calculated.

### 2.4 Thermal properties and crystallinity

Determining the thermal properties of particles was performed by differential scanning calorimetry (DSC) on the Mettler Toledo DSC1 STAR System. All measurements were carried out in nitrogen flow (20 cm<sup>3</sup> min<sup>-1</sup>). The samples were heated from 0 °C to 190 °C at the rate of 10 °C.min<sup>-1</sup>, followed by annealing at 190 °C for 1 min, followed by a cooling scan from 190 to 0 °C (at 10 °C.min<sup>-1</sup>), plus an isothermal step at 0 °C for 1 minute, and finally the second heating scan from 0 to 190 °C (at 10 °C.min<sup>-1</sup>). Melting point temperature (T<sub>m</sub>) as well as the exothermal response, relating to crystallization temperature (T<sub>c</sub>), were obtained from the first heating cycle. From the second heating scan the glass transition temperature region (T<sub>g</sub>) was determined. The degree of crystallinity,  $\chi_c$ , was calculated from the measured heat of fusion ( $\Delta H_f$ ) and crystallization ( $\Delta H_c$ ) according to the following equation:

$$\chi_c = \frac{\Delta H_f - \Delta H_c}{\Delta H_m^0} \times 100\%$$

where  $\Delta H_{0m}$  is the enthalpy of fusion for 100 % crystalline PLA (93.1 J.g<sup>-1</sup>) (Lim et al., 2008.).

## 2.5 Content of D- and L- monomers.

Ratios of both monomers were determined with Kruss P1000 polarimeter at 25 °C using the procedure and data found in Feng et al. [26].

## 2.6 Biodegradation under composting conditions

The method utilized was based on a previously published protocol by Dřfmal et al. [27] with some modifications. Polymer films were cut into 2 mm pieces, then 50 mg of polymer, 5 g of perlite and 2.5 g of dry weight of compost were weighed into each 500 mL biometric flask. The flasks were sealed with stoppers equipped with septa and incubated at 58 °C. Headspace gas was sampled at appropriate intervals through the septum with a gas tight syringe and then injected manually into a GC instrument (Agilent 7890), equipped with Porapak Q (1.829 m length, 80/100 MESH) and 5A molecular sieve (1.829 m length, 60/80 MESH) packed columns connected in series, and a thermal conductivity detector (carrier gas helium, flow 53 mL.min<sup>-1</sup>, column temperature 60 °C). Sampling intervals were operatively adapted to actual CO<sub>2</sub> production and O<sub>2</sub> consumption. Concentrations of CO<sub>2</sub> and O<sub>2</sub> were derived from the calibration curve obtained using a calibration gas mixture with declared composition. The endogenous production of CO<sub>2</sub> by compost in blank incubations was always subtracted to obtain values representing net sample mineralization. From the concentration found, the percentage of mineralization with respect to the initial carbon content of the sample was calculated  $M\% = \text{mgc}/(\text{mswc})$ , where  $M\%$  is the percentage of mineralization,  $\text{mgc}$  is the mass of carbon evolved as CO<sub>2</sub> and obtained from GC analysis,  $\text{ms}$  is the

weight of a polymer sample, and  $\text{wc}$  is the percentage (w/w) of carbon in the polymer investigated. The value of  $\text{wc}$  for the given polymer (50.0 %) was determined on a Flash Elemental Analyzer 1112 (Thermo). In parallel, oxygen concentration was also monitored to provide a control mechanism so as to ensure samples did not suffer from hypoxia. Three parallel flasks were run for each sample, along with four blanks.

## 2.7 Abiotic hydrolysis

Three sets of experiments at different temperatures were performed to determine the extent of PLA hydrolysis in the aquatic environment. Purified PLA powder (80 mg) was suspended in 40 ml of phosphate buffer (0.1 mol.L<sup>-1</sup>, pH 7) in triplicate for each temperature (25, 37, and 58 °C). 1.5 ml aliquots were taken in regular time intervals, centrifuged (10 000 g, 10 min), and the supernatants were analyzed for dissolved organic carbon (TOC 5000A Analyser, Shimadzu). The percentage of hydrolyzed polymer for the given time point was calculated from the amount of dissolved carbon and the initial amount of the material.

## 2.7 DNA isolation, PCR and TGGE conditions

Total DNA was extracted from 0.25 g of compost samples using the PowerSoil® DNA Isolation Kit (MoBio, USA), according to the manufacturer's instructions, involving an initial bead beating step. In the first PCR round, universal bacterial primers  $\text{fD1}$ , and  $\text{rD1}$  (AGAGT TTGAT CCTGG CTCAG, and AAGGA GGTGA TCCAG CC, respectively) were used to amplify nearly the full length 16S rRNA gene [28]. Alternatively, actinomycete specific primers S-C-Act-235-a-S-20-GC, and S-C-Act-878-a-A-19 (CGCGGCCTATCAGCTTGTTG, and CCGTACTCCCCAGGCGGGG, respectively) were used to amplify the V3

to V5 regions of the 16S rRNA gene [29]. Each 25  $\mu$ l PCR reaction contained 12.5  $\mu$ l of GoTaq Green hot start master mix (Promega), 1  $\mu$ l of each primer solution (12.5 pmol), 9.5  $\mu$ l of water for molecular biology and 1  $\mu$ l (5-10 ng) of DNA. All amplifications were carried out on the Piko Thermal Cycler (Finnzymes). The temperature profile for universal primers was as follows: initial denaturation at 94 °C for 5 min.; 30 cycles of 94 °C for 1 min., 55 °C for 1 min., 72 °C for 1 min.; and final extension at 72 °C for 10 min. An alternative program for actinomycete primers went as follows: initial denaturation at 95 °C for 4 min 45 s, followed by 10 cycles of 95 °C for 30 s; annealing temperature stepdowns of 0.5 °C each cycle (from 72 °C to 68 °C); 72 °C for 1 min. The annealing temperature for the final 15 cycles equaled 68 °C with denaturation and extension phases as above. Subsequently, 1  $\mu$ l of the first PCR round was used as a template for nested PCR amplifying of the V3-hypervariable region of the 16S rRNA gene with the primer pair 341fGC and 518r (ATTAC CGCGG CTGCT GG, and CCTAC GGGAG GCAGC AG, respectively), where the GC clamp (CGCCC GCCGC GCGCG GCGGG CGGGG CGGGG GCACG GGGGG) was covalently attached to the 5' end of the forward primer [30]. After 5 minutes of initial denaturation at 94 °C, reactions were subjected to 35 cycles through a TD (touch-down) PCR program (94 °C for 30 s, 61 °C for 30 s and 72 °C for 1 min., followed by a 0.5 °C decrease of the annealing temperature each cycle). After completing 10 cycles in the TD PCR, 25 additional cycles were conducted (94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min.) and 10 minutes of the final extension at 72°C. After each PCR amplification round, the size of the PCR product was verified in 2 % agarose gel. TGGE separation of amplified PCR products was achieved on the TGGE Maxi system (Whatman-Biometra). PCR products (60-

100 ng DNA) from the second amplification process were loaded onto isocratic denaturing gel (8 % acrylamide, 20 % deionized formamide, 50  $\square$  TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4), 2 % glycerol and 8 M urea). A 100-bp DNA ladder (NEB) was loaded as a marker [31]. Electrophoresis was performed at the constant voltage of 130 V for 18 h and the optimal gradient was found to be from 40 to 60 °C. Afterwards the electrophoresis gels were stained with GelStar (Cambrex), according to manufacturer's instructions, and documented. The whole procedure was repeated twice with identical results. Relevant bands were excised, DNA eluted and re-amplified with the same primers, but without GC clamp and send for sequencing.

## 2.8 Microbiological analysis and isolation of the degrading strain and its identification

During biodegradation experiments, counts of total heterotrophic bacteria, actinomycetes, and fungi were monitored on tryptone yeast agar (HiMedia), glycerol nitrate agar (Pitt, 1979), and yeast glucose chloramphenicol agar (HiMedia), respectively, after 7 days at 58 °C.

To isolate PLA degrading strains, one gram of the material from PLA biodegradation experiments was suspended in 9 mL of suspension media [32] and shaken for 15 min. The suspension was then spread in different dilutions on mineral agar media with PLA1 suspension [33] as the sole carbon source and incubated for five days at 58 °C. Dry white colonies were isolated to purity and their growth on PLA verified. Almost the entire sequence of its 16S rRNA gene was sequenced (GeneBank accession number: JX255731) and compared with the public database (GenBank).



### 3. Results and discussion

#### 3.1. Characterization and processing of PLA samples

For all four PLA samples used in this study, properties that could be critical for the interpretation of relatively difficult and time-consuming biodegradation experiments were investigated. Measurements with gel chromatography proved that selected materials had their MW well distributed over a relevant interval and the polydispersities of their MW distribution curves were comparable for three of the four materials but higher in the case of PLA2 (Table 1). Thermal analysis can be efficiently used to estimate the crystalline part of polymer materials. It is believed that the amorphous regions are biodegraded preferentially and some authors interpreted an increase in crystallinity during incubations as the proof of biodegradation [34,35]. DSC measurements were performed for all polymer samples and their forms and degrees of crystallinity were deduced (Table 1), with the exception of the thin coating sample form, for which the analysis was not feasible. In principle, crystallinity could significantly be influenced by the processing of samples. For the investigated sample forms, crystallinities were roughly comparable and the crystallinities of powder forms were always lower than film forms, probably reflecting the shorter time available for crystallite formation during the powder form preparation. Crystallinities of PLA1, PLA3 and PLA4 were roughly comparable with somewhat higher values found with PLA2.

The properties of PLA are influenced by the content of D- monomer. Even a low content of the D monomer could markedly decrease crystallinity thus promote the abiotic hydrolysis [12]. It seems that D monomer content do not also inhibit the enzymatic hydrolysis, which in contrary

can be further supported with the lower crystallinity [36]. To address this aspect the stereochemistry of incorporated monomers was determined by polarimetry (Table 1), and it was found that the D monomer contents were comparable in all PLA samples.

#### 3.2. Biodegradation of PLA samples in compost

The described PLA samples were incubated for about 100 days under composting conditions at 58 °C (Fig. 1). The initial lag phase of about 20 days duration at the beginning of biodegradation could clearly be distinguished for two higher MW samples, whereas it was apparently missing with the lowest MW PLA. For PLA2, the lag phase could not be clearly observed but the curves exhibited some level of acceleration at about day 25. It could be concluded that, for PLA with a MW of over approximately 60 kg mol<sup>-1</sup>, this higher MW caused significant retardation of the biodegradation onset. The curves presented also reflected the type of the sample. Whereas for the low MW sample all its curves are perfectly parallel and the lower specific surface area of the film sample did not limit biodegradation, for higher MW film samples biodegradation of this sample type was markedly retarded. Interestingly, in the case of the highest MW sample, the higher specific surface area of the powder sample did not represent a significant advantage and biodegradation was almost identical to that of the film form. Somewhat specific was the behavior of samples prepared as a thin coating on the inert porous material perlite. The biodegradation of this particular sample type was the fastest with all MWs tested, and even for the highest MW PLA, it was significantly less retarded at the beginning. One can speculate that the thin coating form provides an additional surface of highly porous perlite for microbial colonization, and thus promote faster biodegradation, and/or this

form has a significantly higher specific surface even over the powder form.

The same data as in Fig. 1 are also represented in Fig. 2 ABC, but with a different arrangement that makes possible to compare PLA samples with different MWs for individual sample forms. It is evident that the biodegradation rate was dependent on MW for all sample forms, and that higher MW samples exhibited retardation during the initial phase, which could be described as a lag phase or autocatalytic onset of biodegradation. In later phases, biodegradation rates decreased with increasing MW for all sample forms. As has already been mentioned, the thin coating sample form always exhibited the highest biodegradation rates, which is even more evident in this representation of the data. The initial retardation appeared gradually more pronounced with increasing MW for all sample forms.

The retardation of higher MW PLA biodegradation could be caused by several factors. One such possible explanation comprises the idea that low MW PLA better supports the growth of degrading microorganisms, and eventually the induction of necessary specific enzyme systems. Not possessing this potency, high MW PLA biodegradation could suffer from the slower onset of enzyme activities and initially only a slowly growing number of specific degraders. In such a case, the relatively limited addition of low MW PLA could induce the enzymes and the growth of the degraders and significantly accelerate the biodegradation of high MW PLA. It was decided to test this hypothesis in a separate experiment (Fig. 2D). Mixtures of PLA1 and PLA4, with weight ratios from 5/95 to 20/80, were monitored in the composting experiment along with pure PLA1 and PLA4 samples, all in powder form. The data shows that the additions up to 20% of PLA1 did not significantly enhance the biodegradation of high MW PLA4; the acceleration during the first 30 days corresponded to the added

fraction of low MW PLA1 only. Based on these results, the above hypothesis that the retardation is caused by the low number of initially slowly growing degrading microorganisms or low induction of specific enzymes by high MW PLA seemed less likely.

### 3.3 Abiotic hydrolysis of PLA

Both abiotic and enzymatic hydrolysis can participate on PLA biodegradation. To estimate the importance of the abiotic hydrolysis for the particular PLA samples in the study an experiment was set up where the hydrolysis in aqueous environment in the presence of a microbial growth inhibiting substance (NaN<sub>3</sub>) was followed as the total soluble organic carbon released to aqueous environment. The effect of elevated temperature on PLA hydrolysis could be crucial for the good biodegradability of PLA under composting conditions [11] and was tested as well (Fig. 3). It is evident that the hydrolysis of all PLA samples was markedly accelerated with temperature whereas at individual temperatures the hydrolysis rates followed MW of PLA samples with lowest MW PLA as the fastest.

Interesting information comes from the comparison of data from biodegradation in compost at 58 °C and from hydrolysis at 58 °C (Fig. 1). It is evident that the hydrolysis curve of the material powder form almost copies the same material biodegradation curve. In composting experiment at 100% relative humidity hydrolysis rate should be comparable. Under such assumption the results suggest that the abiotic hydrolysis controlled the rate of biodegradation in the system described and the observed initial retardation of biodegradation can be a result of suggested autocatalytic nature of PLA hydrolysis [9] and consequently the role of a specific enzymatic hydrolysis of PLA ester bond could only be limited.

### 3.4 Microbial community during PLA biodegradation

In theory, the differences in biodegradation between different PLA samples in the study could be explained by differences in the participating microorganisms. For example specialized strains could be necessary to decompose high MW PLA.

Firstly, a classical approach was employed to isolate the degrader from the composting experiments. One visually identical strain intensively growing on mineral agar with PLA suspension as the sole source of carbon and energy was successively isolated from biodegradation experiments with all different PLA samples. The strain was identified by 16S rDNA sequencing as highly similar to *Thermopolyspora flexuosa* DSM 43186 (accession number FN667472.1, 98% identity) belonging to the taxonomical group of thermoactinomycetes and designated *Thermopolyspora flexuosa* FTPLA (GenBank accession number JX255731).

To gain further information about the microbiology of PLA biodegradation a cultivation independent method was employed. Temperature gradient gel electrophoresis (TGGE) can visualize microbial communities during the composting experiments and their temporal dynamics. Furthermore, separated DNA species from microorganisms present can subsequently be sequenced, and thereby the taxonomy of individual microorganisms revealed. It must be admitted that the method is capable of identifying the major members of the community only and some minor but possibly crucial microorganisms need not to be recognized, but still it represents an excellent tool for comparing microbial community in different composting experiments and its evolution in time. PCR generated DNA fragments in selected time points of composting experiments with the powder form of PLA1 and PLA4 (Fig. 2B) were TGGE separated and visualized (Fig.

4A). Without explaining the method in details, it may be simply expected that each band represents an individual bacterial strain. Universal bacterial PCR primers were used for the left half of the gel, so the signals of all bacteria present should be visible. This approach did not revealed any signals of candidates for the specific PLA degrading bacteria since all the signals, which eventually increased their intensity, were also present in blank incubations without PLA. Considering the above described results of PLA degrading strain isolation and literature data suggesting the importance of actinobacteria in PLA degradation under composting conditions [37] it was decided to focus on this particular group of microorganisms. Microbiological analysis proved that thermophilic actinobacteria counts increased markedly during the composting experiment (from 106 to 108 CFU in 1 g of the culture dry weight), whereas total thermophilic bacteria counts were relatively stable (about 108 CFU in 1 g of the culture dry weight) and fungi were not present at all at the given temperature. Primers specific for actinobacteria were used in second TGGE analysis (Fig. 4A, right) so that only the signals of bacteria from this taxonomic group would be visible and the eventual changes highlighted. Using actinomycete specific primers, one of the bands (band 1) was markedly intensified in connection with PLA biodegradation and its sequencing proved that it is identical to the above described strain *Thermopolyspora flexuosa* FTPLA. To obtain more detailed information about the actinomycete community, especially at the beginning of degradation as well as on the lag phase phenomenon, DNA from another composting experiment was isolated and analyzed using TGGE with actinomycete specific primers (Fig. 4B). It is clearly visible that band 2 (identical according to its sequence to band 1 from Fig. 4A) is absent at day 0 but had already appeared at day 6 in the presence of all the PLA types



investigated, and intensified at the end of the lag phase when acceleration of the biodegradation began. Accordingly, the band intensity corresponded to the mineralization curves of different MW PLA samples (Fig. 2B). It is thereby evident that the acceleration of PLA biodegradation at the end of the lag phase occurs simultaneously with the multiplication of the degrader. Bands 3 and 4 were more difficult to interpret. For PLA1, they also intensified during the incubation period; in incubations with higher MW PLA2 and PLA3, they are present from the beginning and they could also be clearly recognized (proved with sequencing) in the blank incubation. Thus, it might be concluded that bacterial strains corresponding to these microorganisms probably were not primary PLA degraders. The TGGE profiles of blank incubations clearly documented the different structure of bacterial community in the absence of PLA. Some of the intensive bands could be identified and were found different from those in PLA presence, identification of selected bands is provided in the Figure 4 caption.

In sum, despite of the differences in PLA samples investigated especially very different MW, all PLA samples were biodegraded by the same principal degrader, which was isolated and identified.

#### 4. Conclusions

Biodegradation of four PLA grades with different MW and different forms of the samples was investigated. Among PLA materials the rate of biodegradation followed the pattern of increasing MW with characteristic lag-phase or autocatalytic shape of the curves apparent for higher MW samples. The specific surface area also proved to be an important factor promoting fast biodegradation especially with higher MW samples. Material properties of the PLA samples did not seem to influence the composition of

microbial communities degrading the samples. The same organism, *Thermopolyspora* strain, was always discernible as the principal degrader with all PLA samples, where the increase of its quantity was simultaneous with the acceleration of biodegradation at the end of the lag phase. The addition of limited amount of low MW PLA did not accelerate biodegradation of high MW PLA so it seems that the biodegradation is not limited with the number of specific degraders and/or the induction of specific enzymes. The most important finding, in accordance with the above conclusion points, appears to be the observation that the course of the biodegradation was quasi identical with the course of abiotic hydrolysis for all PLA grades investigated so the abiotic hydrolysis, and not the enzymatic one, was the major depolymerization mechanism and the rate controlling step of the biodegradation process.

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TABLE 1. Properties of various PLA samples and the thermal properties of their individual sample forms

Sample	$M_w$ g.mol <sup>-1</sup>	$M_w/M_n$	L-lactide %	Form	$T_m$ °C	$T_g$ °C	$\chi_c$ %
PLA 1	34000	2	95.5	Film	144.4	34.4	39.5
				Powder	151.5	50.9	29.7
PLA 2	61000	2.9	94.6	Film	165.3	50.8	58.5
				Powder	162.8	54.3	41.1
PLA 3	109000	2.3	95.8	Film	168.3	55.8	51.6
				Powder	168.0	55.5	35.4
PLA 4	160000	1.9	96.0	Film	168.3	54.5	40.9
				Powder	167.3	55.1	33.9

$M_w$ , weight average molecular weight;  $M_w/M_n$ , polydispersity;  $T_m$ , melting point temperature;  $T_g$ , glass transition temperature;  $\chi_c$ , degree of crystallinity.

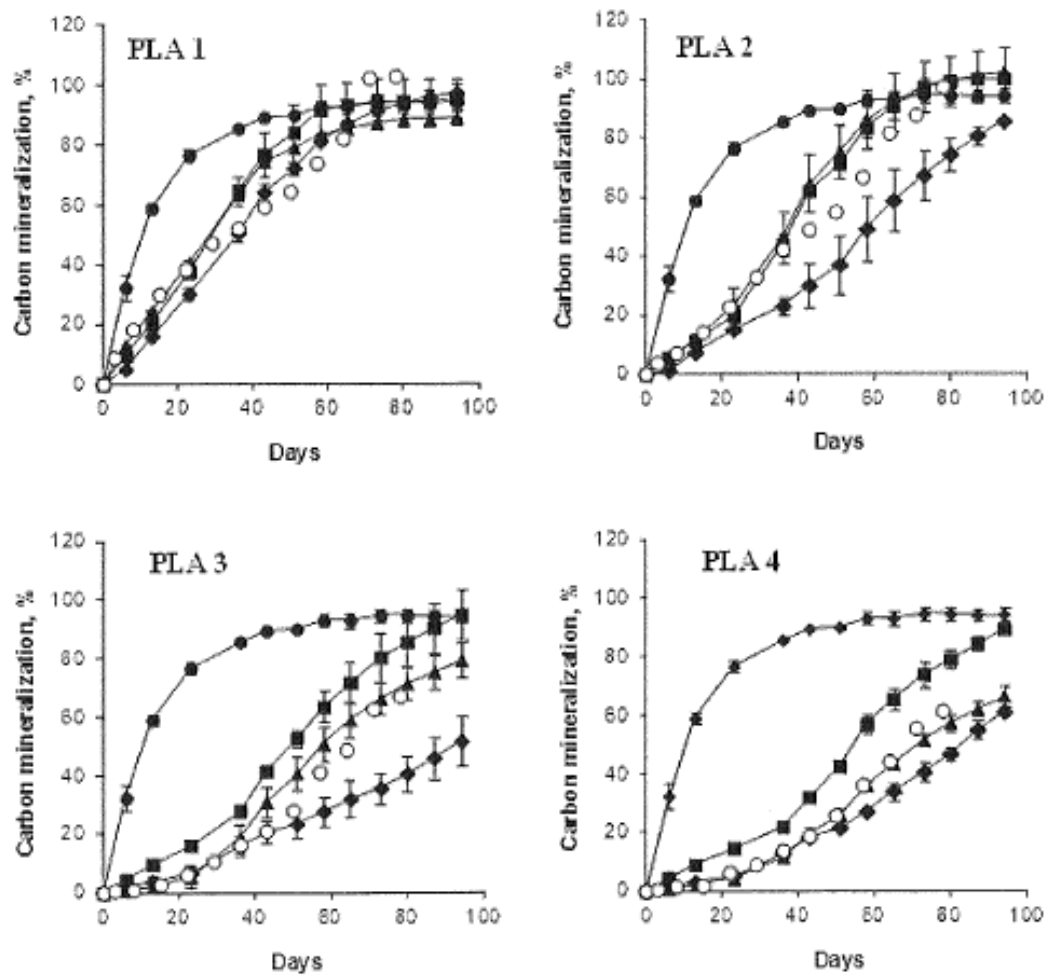


Figure 1. Biodegradation of PLA with various MWs in the compost experiment, comparison with the abiotic hydrolysis of powder form PLA. Comparison of sample forms with different specific surface areas. ■ thin coating; ▲ powder; ◆ film (100  $\mu\text{m}$ ); ● cellulose (reference compound); ○ abiotic hydrolysis of the powder form sample. Error bars correspond to twice standard deviation (n=3).



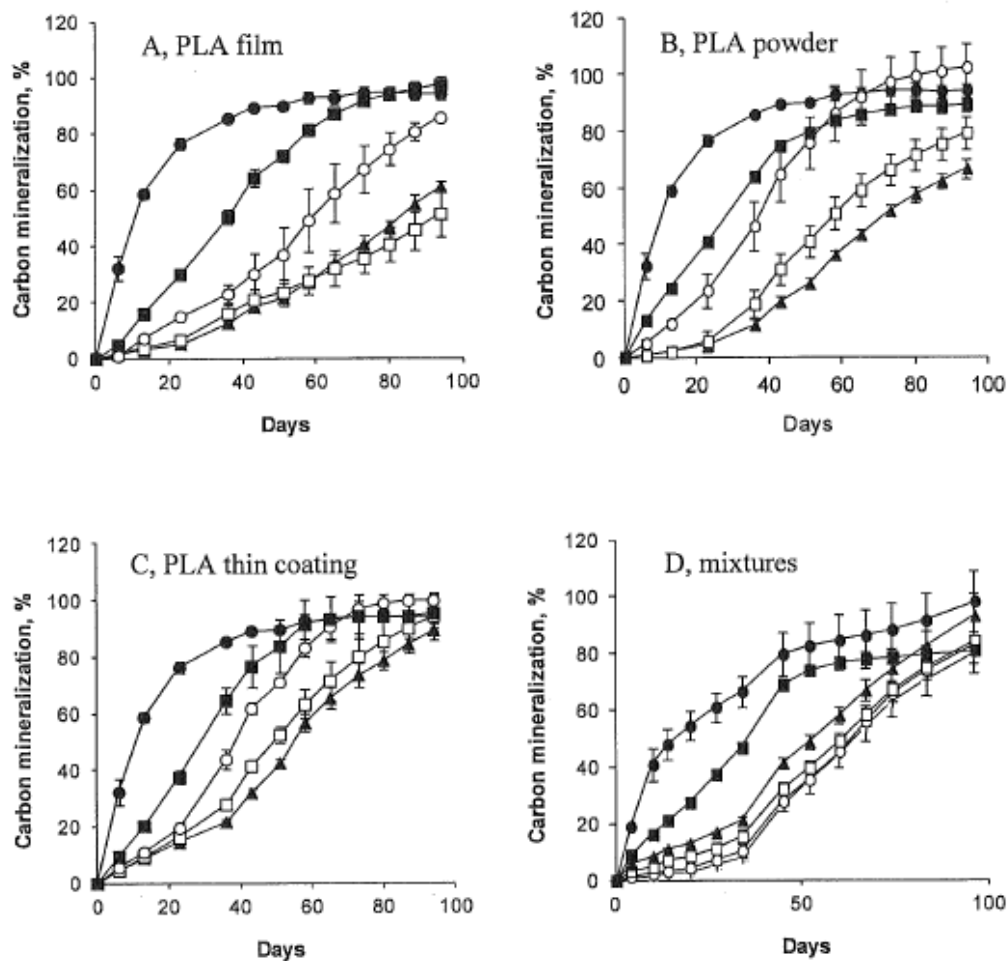


Figure 2. Panels A, B, and C: Biodegradation of PLA sample forms with different specific surface areas in the compost experiment. Comparison of PLA samples with various MWs. ■, PLA1; ○, PLA2; □, PLA3; ▲, PLA4. Panel D: Biodegradation of PLA1 and PLA4 powder mixtures in various ratios in the compost experiment. ■, 100% PLA1; ▲, 20% PLA1 + 80% PLA4; □, 10% PLA1 + 90% PLA4 ; ○, 5% PLA1 + 95% PLA4; + 100% PLA4. ●, cellulose (reference compound). Error bars correspond to twice standard deviation (n=3).

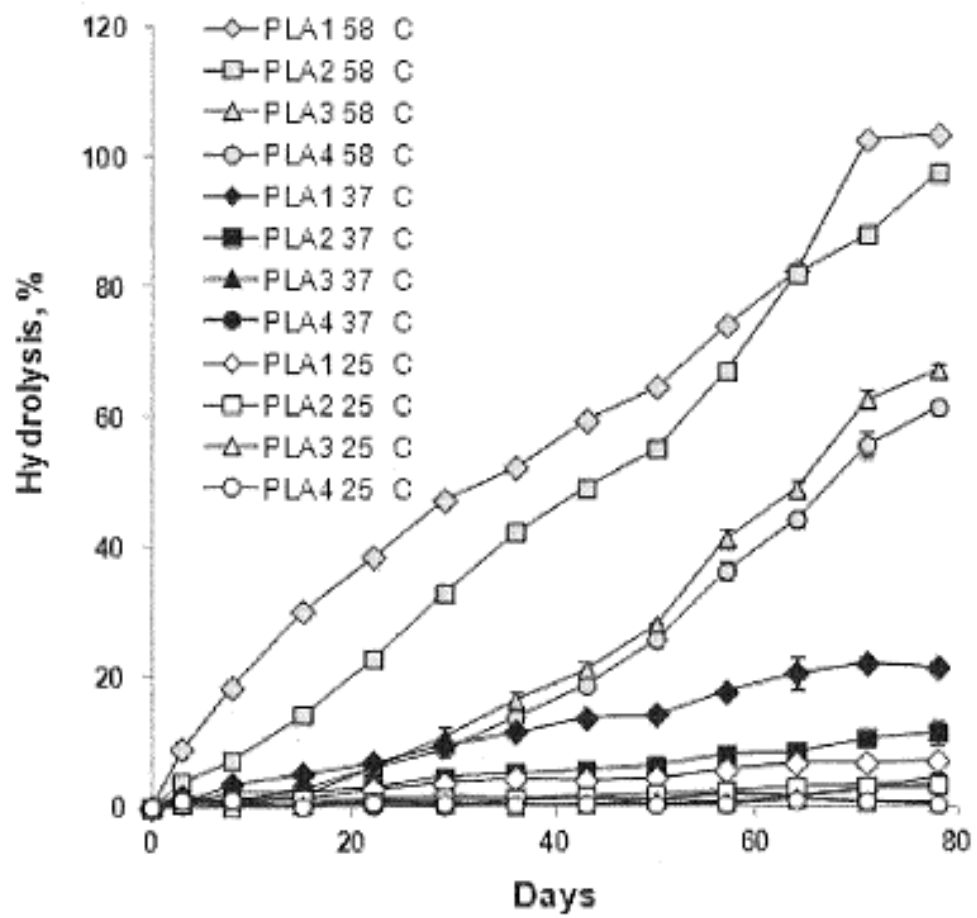


Fig. 3. Abiotic hydrolysis of different PLA grades and forms in aqueous environment at various temperatures.



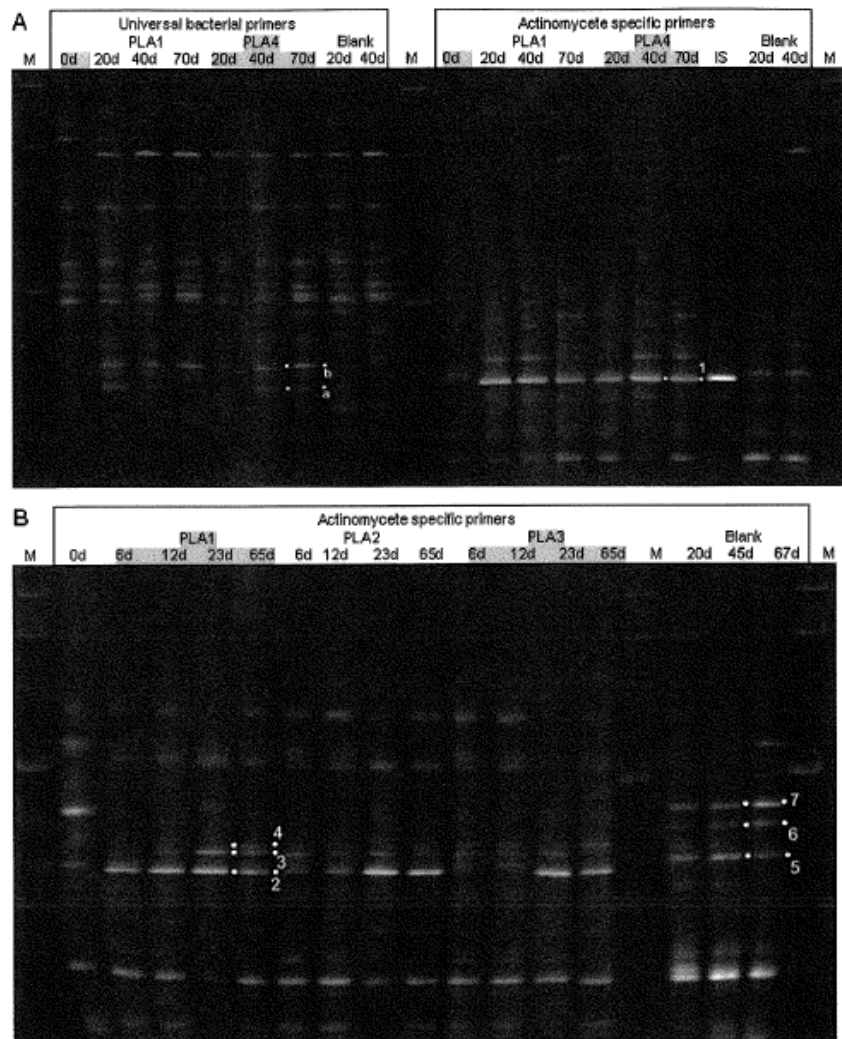


Figure 3. TGGE profiles documenting temporal dynamic of microbial communities during biodegradation of PLA with different MW. Either the total bacterial community (Universal bacterial primers) or actinomycete community only (Actinomycete specific primers) were monitored. Numbers featuring "d" denotes the number of days from the beginning of the biodegradation experiment. IS: An isolated degrading strain designated as *Thermopolyspora flexuosa* FTPLA. Blank: The blank incubation was conducted under identical conditions but without PLA. Identification of TGGE bands (band number, sequence number, organism name, percentage of identity): 1, JN188948.1, *Thermopolyspora* sp. YIM 75076, 97%; 2, JN188948.1, *Thermopolyspora* sp. YIM 75076, 97%; 3, *Thermomonospora* sp. S22-23, 98%; 4, AM932264.1, *Thermomonospora* sp. S22-23, 98%; 5, GU412146.1, *Atopobium* sp., 93%; 6, JN187087.1, *Serinicoccus* sp. BSJ2, 91%; 7, HQ688671.1, *Thermobifida fusca* DY-3, 86%.

## Use of temperature gradient gel electrophoresis for the investigation of poly(vinyl alcohol) biodegradation

L. Husarova, J. Ruzicka, H. Marusincova and M. Koutny

**Abstract**— The application of temperature gradient gel electrophoresis (TGGE) as a culture-independent method to monitor changes in the composition of bacterial community during PVA degradation under denitrification conditions is reported. TGGE was used to separate DNA fragments after PCR amplification from total DNA extracted from the acclimated denitrifying sludge with universal bacterial primers targeted the region of the 16S rRNA gene. TGGE proved to be useful and enable to visualize potential degraders and their time dynamics during the biodegradation experiment.

**Keywords**— poly(vinyl alcohol), waste water treatment sludge, temperature gradient gel electrophoresis (TGGE).

### I. INTRODUCTION

Poly(vinylalcohol) (PVA) is water-soluble synthetic polymer which has a wide range of application in industrial sectors. Its properties comprise viscosity, flexibility, film forming, dispersing power and adhesive strength. Due to these properties PVA is mainly used in sectors such food, chemical, paper and textile industries. This polymer belongs as well as Polyvinylethylen (PE) to group of biological degradable plastics. Among the most common microorganisms degrading PVA *Pseudomonas* or *Sphingomonads* can be found. PVA is biodegradable under aerobic conditions [1] and partially in anaerobic environment [2], [3], but in latter case a longer time for degradation of PVA is apparently needed.

DNA based microbial community fingerprinting techniques like temperature gradient gel electrophoresis (TGGE) or most often denaturing gradient gel electrophoresis (DGGE) have been so far extensively applied to describe the structure of procaryotic communities [4], [5]. They allow simultaneous processing of a larger number of samples and provide an estimate of diversity independent to conventional cultivation techniques. TGGE and DGGE are based on the separation of DNA fragments of the identical size differing in their

nucleotide sequence [6], [7]. Sequence separation occurs in acryl amide gels with a denaturing gradient, where each fragment migrates and finally stops at its melting point. Melting point is directly related with its guanine-cytosine (GC) content [7], [8].

Our main goal was to adapt the TGGE technique to monitor changes in the composition of bacterial community during PVA degradation under denitrification conditions.

### II. MATERIALS AND METHODS

#### A. Degradation experiments.

Denitrifying sludge was used from a municipal waste water treatment plant. The sludge was kept under nitrogen until its use. PVA (100 mg.l<sup>-1</sup>) and KNO<sub>3</sub> (1000 mg.l<sup>-1</sup>) were dissolved in mineral medium and this solution (culture medium) was purged with a stream of nitrogen. The mineral medium contained (mg.l<sup>-1</sup>): 50 mg MgSO<sub>4</sub>.7H<sub>2</sub>O; 30 mg Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>.6H<sub>2</sub>O; 10 mg CaCl<sub>2</sub>.2H<sub>2</sub>O; 50 mg NH<sub>4</sub>Cl, 0.086 mg MnSO<sub>4</sub>.5H<sub>2</sub>O; 0.114 mg H<sub>3</sub>BO<sub>3</sub>; 0.086 mg ZnSO<sub>4</sub>.7H<sub>2</sub>O; 0.074 mg (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>; 0.05 mg Co(NO<sub>3</sub>)<sub>2</sub>.6H<sub>2</sub>O; 0.08 mg CuSO<sub>4</sub>.5H<sub>2</sub>O; 181 mg K<sub>2</sub>HPO<sub>4</sub> and 1912 mg Na<sub>2</sub>HPO<sub>4</sub>.12 H<sub>2</sub>O

The acclimated denitrifying sludge was centrifuged at 4600 RPM for 10 minutes. Then the wet sediment (1g.l<sup>-1</sup>) was suspended in culture medium. This suspension or its dilutions in culture medium (10<sup>-1</sup>, 10<sup>-2</sup> and 10<sup>-3</sup> g l<sup>-1</sup>) were filled in the test gasproof flasks (110 ml) and bubbled through with nitrogen again. Test flasks were incubated at 25°C and stirred at 250 RPM. Samples were taken by injection syringe and centrifuged (10 000 RPM, 20 minutes). The wet sediments were saved for analysis of DNA and concentrations of PVA in supernatants were determined by a iodometric method [9].

#### B. DNA extraction and PCR amplification

DNA was isolated from using a commercial DNA extraction kit according to the instructions of the manufacturer (Mo Bio). Eubacterial 16S rRNA gene was amplified with universal primers rD1 and fD1 [10]. In the second nested PCR the primer pair 341f and 518r (5'-ATT ACC GCG GCT GCT GG-3' and 5'-CCT ACG GGA GGC AGC AG-3') with a GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') [11] attached to the forward

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L. Husarova, J. Ruzicka, H. Marusincova, M. Koutny are with Department of Environmental Protection Engineering, Faculty of Technology, Tomas Bata University in Zlin, 762 72 Zlin, Czech Republic (corresponding author, phone: 00420-604-559-681; e-mail: mkoutny@ft.utb.cz).



primer was used. Hot-start PCR was carried out in a 25  $\mu$ l reaction mixture containing 12.5  $\mu$ l of GoTaq green hot start master mix (Promega), 1  $\mu$ l of each primer solutions (12.5 pmol), 8.5  $\mu$ l of water and 2  $\mu$ l (5-10 ng) of bacterial DNA solution. Samples were amplified in Piko Thermal Cycler (Finnzymes) using the following program: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min. Subsequently, 1  $\mu$ l of the first PCR product was used as a template for nested PCR, with the primer pair 341fGC and 518r. Temperature program consisted of 1 min at 94°C and 30 cycles of 1 min at 94°C, 1 min annealing at 55°C, 1 min extension at 72°C and final extension at 72°C for 10 min. The size and amount of the PCR products was confirmed by agarose gel electrophoresis.

### C. TGGE conditions

TGGE Maxi system (Whatman-Biometra) was used for the separation of nested PCR products. Electrophoresis was performed in 1 mm thick polyacrylamide gel (at constant current 30mA). Denaturing gels (8% acrylamide, 20% deionized formamide, 1x TAE (Tris-acetate-EDTA buffer), 2% glycerol and 8M urea) were made with 2x TAE buffer. After it, the gel was polymerized by adding 110  $\mu$ l of N,N,N',N'-tetramethylethylenediamine (TEMED) and 50  $\mu$ l of 10% ammonium persulfate (APS) to 50 ml of the gel components mixture. Then, the gel was let to polymerize at least for 2 hours. Consequently, two to five micro liters of PCR products (60-100 ng DNA) were loaded into each well and run at 130 V for 18h. For our purpose, the optimal thermal gradient was found to be from 40 to 60°C. Finally, gels were developed by silver staining following a modification of a protocol [12], [13]. The gel was fixed in the solution of 10% (v/v) ethanol and 0.5% acetic acid for 1h. Then, it was impregnated with 0.2% (wt/v) silver nitrate for 5 min. After two thorough washes with distilled water, the freshly prepared developing solution containing sodium hydroxide (15 g.l<sup>-1</sup>) and 37% HCOH (2 ml.l<sup>-1</sup>) was poured onto the gel. After 5 min the solution was removed and the gel was dried. The gel image was then taken using a digital camera (Syngene).

### III. RESULTS AND DISCUSSION

It was found in some previous experiments that PVA is degraded under anaerobic denitrification conditions by the bacterial community present in the sludge from denitrification compartment of the local municipal waste water treatment plant. To investigate the bacterial community and visualize its dynamics TGGE technique was chosen and applied to monitor changes in the community during a biodegradation experiment. Denitrification supporting media containing PVA as sole source of carbon and energy were inoculated with series of dilutions of the bacterial community from the waste water sludge previously acclimated under denitrification conditions with PVA.

Course of PVA biodegradation was followed in these cultures for about 30 days and the results are depicted in figure 1.

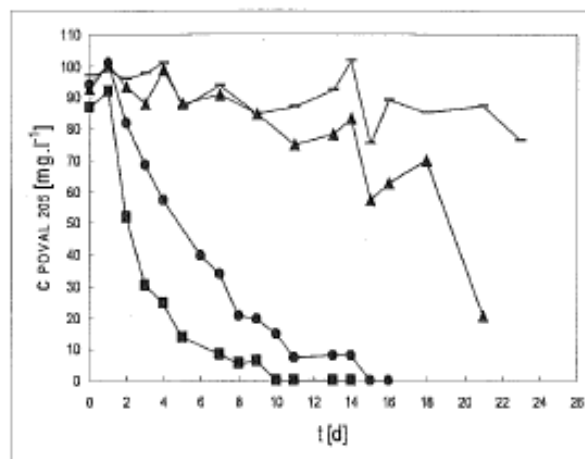


Figure 1. Monitoring of gradual decrease of PVA during denitrifying conditions, culture inoculum with  $\blacksquare$  1 g.l<sup>-1</sup>,  $\blacktriangle$  0.1 g.l<sup>-1</sup>,  $\bullet$  0.01 g.l<sup>-1</sup>,  $\blacklozenge$  0.001 g.l<sup>-1</sup> of sludge.

It is evident that for two highest inoculations PVA was consumed very fast without any lag phase on the beginning of the observation period. However for the medium inoculation density (0.01 g of sludge per liter of medium) the rate was strongly retarded with an evident lag phase. For the highest dilution the active microorganisms seemed to be diluted out. Samples taken from cultures inoculated with 0.1 and 0.01 g of the sludge per liter were chosen for further experiments.

Whole bacterial DNA was isolated from the samples taken in selected days of the degradation experiment and subsequently fragments of bacterial 16S rDNA gene were PCR amplified and resolved with help of TGGE. Patterns of bands visible in individual lines represent bacterial communities in a given day of incubation (Fig. 2.). Virtually, each band should represent an individual bacterial strain. For both dilutions samples at beginning (day 0), in the middle and at the end of PVA consumption were investigated. Main attention was paid to a search for an intensive band not present or having weak appearance on the beginning of the experiment (day 0) and becoming intensive during the phase of intensive biodegradation. Such band was identified and designated in Figure 2 as "d". Additional new bands appeared in the late phase of biodegradation (days 6 or 21 and bands a, b, c, f). We speculated that respective bacterial strains probably utilized some degradation products of PVA formed by the action of the strain related to the band "d" which we assumed was the principal degrader of PVA in the described experiment. Some changes in band intensities (bands e, g) remain more difficult to interpret.

The study proved that TGGE is suitable and could be extremely helpful method in further investigation of PVA biodegradation.

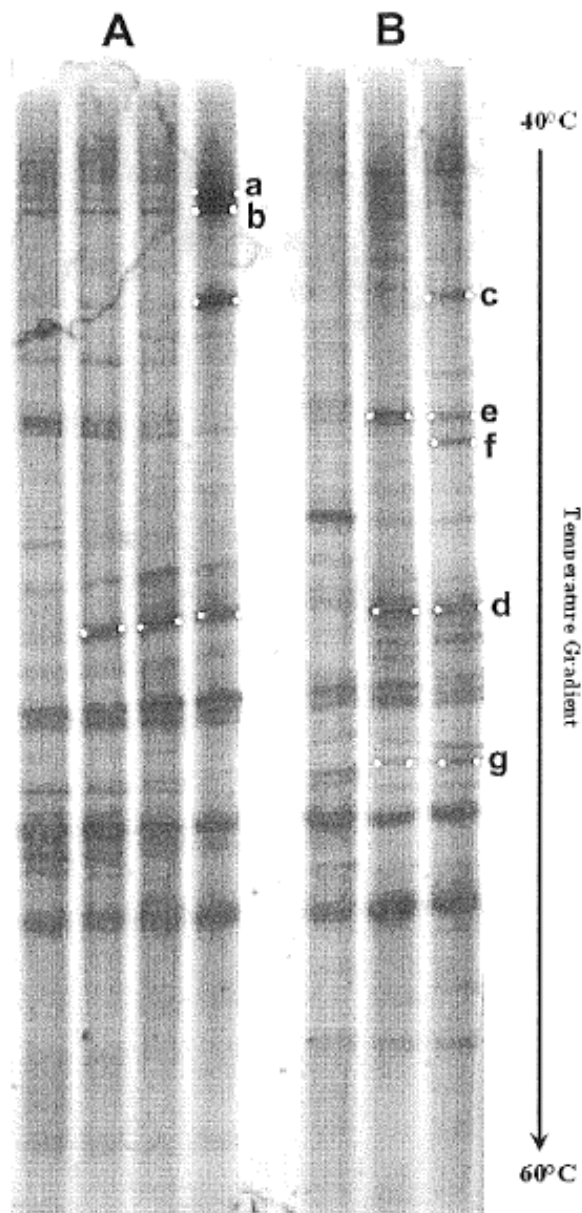


Figure 2. Temperature gradient gel electrophoresis profile of PCR products of 16S rDNA of changing bacterial communities during denitrifying biodegradation of PVA, A (cultured inoculum with  $0.1 \text{ g.l}^{-1}$  of sludge), B (cultured inoculum with  $0.01 \text{ g.l}^{-1}$  of sludge).

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## Příloha P6: Identification of several factors affecting biodegradation of aromatic-aliphatic copolyester (plnotextový příspěvek na konferenci)

Development, Energy, Environment, Economics

# Identification of several factors affecting biodegradation of aromatic-aliphatic copolyester

Petr Stloukal, Jiri Jandak, Lucie Husarova, Marek Koutny, Sophie Commereuc and Vincent Verney

**Abstract** — Aromatic-aliphatic polyesters aspire to be an eco-friendly replacement of conventional non-biodegradable polymers. Biodegradation of studied aromatic-aliphatic copolyester was estimated in soil and compost. The material was introduced into the tests in four forms differing above all by their specific surface. The copolyester in principle proved to be biodegradable under compost conditions. Biodegradation was also tested in five well selected and characterized agricultural soils. Here its biodegradability was not found to be significant. The specific surface of sample specimen was shown to be an important factor affecting the rate of biodegradation in compost, where biodegradation sample forms with higher specific surface was significantly accelerated over sample specimens with low specific surfaces.

**Keywords** — biodegradation, composting, copolyester, mulching film, soil.

### I. INTRODUCTION

PLASTIC litter represents not only a serious environmental but also a social problem. Public is greatly alarmed at the omnipresence of plastic litter in the environment. The problem is particularly striking in less developed countries where functioning of waste management systems is insufficient. It should be pointed out that still the most recommended way of plastic waste treatment remains recycling but for many applications this approach tend to be economically not feasible due to the high cost of collection, separation and cleaning of the raw material. Plastic films are also still more extensively used in agriculture as mulching films. Here also the collection and especially cleaning is problematic.

In the past years growing interest is paid to the development of biodegradable polymers that could be able to replace conventional polyolefin films in many applications including mulching films and packaging applications [1,2]. The particularly perspective branch of such materials is represented by synthetic copolyesters with content of aromatic and aliphatic components [3,4]. Changing the component ratio one

is able to balance processing properties of the copolyester and its biodegradability [5,6,7]. In principle, aliphatic parts of the copolyester favor biodegradability of the polymer [8]. Several these materials were already commercialized and they are available on the market [9].

However biodegradation of such materials was not extensively enough studied especially regarding different conditions where and how the biodegradation should occur.

The present study deals with one particular example of the described materials. We wanted to show that its biodegradation can be greatly affected by the form of the sample specimen and can differ in various environments.

### II. MATERIAL AND METHODS

#### A. Material

The polymer used throughout the study was aromatic aliphatic copolyester containing units of terephthalic acid, adipic acid and 1,4-butane-diol (molar ratio 22:28:50, respectively).

#### B. Polymer processing

Raw polymer in the form of pellets was processed to obtain different forms of the polymer with different specific surfaces.

*Films.* Films were prepared by compression molding at 140°C. According to the inserted steel frame films of 100 µm and 300 µm were prepared.

*Powder.* Chloroform solution of polymer (50 mg/ml) was poured into four volumes of ethanol and the mixture was vigorously stirred. Obtained precipitate was filtered out and dried on air. Resulting powder was characterized by microscopy (Fig. 1A, 1B). There were of irregular shape with diameters up to 500 µm. Electron microscopy with higher magnification revealed the important specific surface of the particles (Fig. 1B).

*Thin coating on inert surface.* Calculated volume (4 ml) of the chloroform solution of polymer (50 mg/ml) was applied on the surface of pre-weighed porous inert material (perlite, 7 g) and stirred. Then the solvent was stripped out with air leaving the polymer coating on the surface of perlite.

#### C. Biodegradation experiment

Three components were weighed into 500 ml biometric flasks: polymer samples (200 mg), mature compost or any of tested soils (5 g of dry weight) and perlite 7 g. Biometric flasks were equipped with septa on stoppers. Sample flasks

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P. Stloukal, M. Koutny and L. Husarova are with Environmental Protection Engineering, Faculty of Technology, Tomas Bata University in Zlin, 762 72 Zlin, Czech Republic (corresponding author, phone: 00420-604-559-681; e-mail: mkoutny@ft.utb.cz).

J. Jandak is with Faculty of Agronomy, Mendel University in Brno, 613 00, Czech Republic.

S. Commereuc and V. Verney are with the Clermont Université, UBP, Laboratoire de Photochimie Macromoléculaire, F-63000 Clermont-Ferrand.

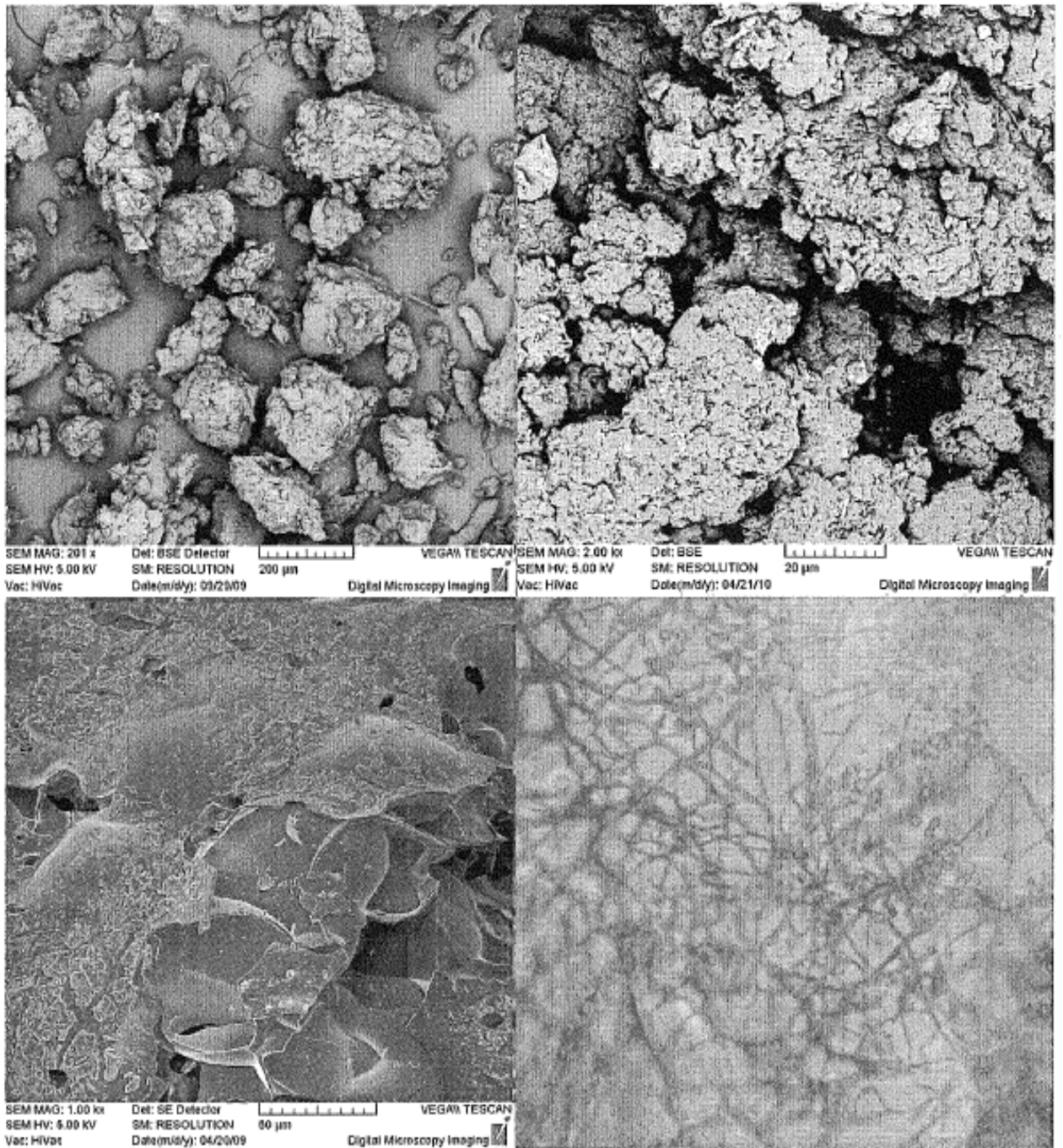


Fig. 1. A, electron microscopy of precipitated polymer powder (200×); B, electron microphotography of polymer powder at higher magnification (2000×) showing high specific surface of the powder particles; C, electron microphotography of polymer coating (upper left part of the microphotograph) on the surface of perlite (1000×); D, optical microscopy of bacterial filaments on the surface of 100 μm polymer film after incubation in compost (1000×), filaments were stained with carbol-fuchsin and then observed under immersion oil, endospores typical for thermoactinomycetes are apparent as tiny nodules, the picture could not be made well focused everywhere because the film was not perfectly flat.



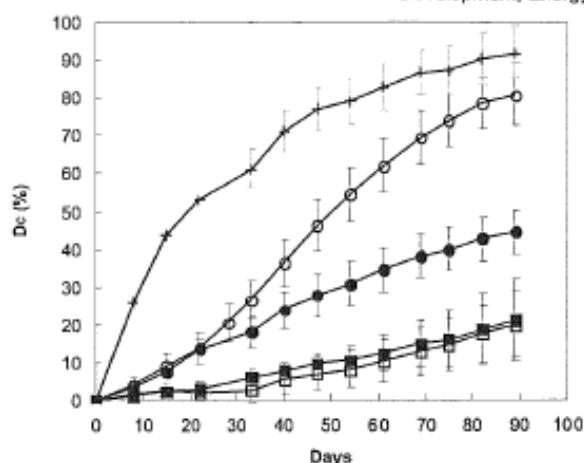


Fig. 2. Biodegradation of different forms of the polymer in compost experiment. □, thin film (100  $\mu\text{m}$ ); ■, thick film (300  $\mu\text{m}$ ); ○, powder; ●, thin coating; +, cellulose (reference compound). Error bars correspond to twice standard deviation ( $n=3$ ).

#### D. Microscopy

For optical microscopy of the surface biofilm the polymer film fragment was fixed with 4% formaldehyde (overnight at 4°C) and then stained with carbolfuchsin. For scanning electron microscopy a polymer sample was coated with thin gold/palladium layer and observed in VEGA LMU (Tescan, CZ) instrument.

### III. RESULTS

Investigated copolyester was designed to be a biodegradable material with potential applications in agriculture. Preliminary experiments showed (data not presented) that biodegradation of relatively thick compression (100  $\mu\text{m}$ ) molded polymer films appeared to be relatively slow. We decided to test whether this phenomenon was caused by low specific surface of the films. To answer the question we prepared two other forms of the polymer with higher specific surfaces, solvent precipitated powder of the polymer and deposition of the polymer on high specific surface inert material (perlite). Furthermore we intended to compare the polymer biodegradation under composting conditions and its biodegradation in a set of selected soils.

#### A. Biodegradation in compost

Results from compost experiment are well illustrated by Figure 2. Samples in forms of thin and thicker films behaved almost equally and end up with about 20% carbon mineralization after 90 days of incubation. Final values must be evaluated as relatively poor and hardly acceptable in an industrial composting plant.

However for the two forms of sample with higher specific surfaces the results are clearly more optimistic with about 45% carbon mineralization in the case of polymer coating on teh material with high specific surface (perlite). The fastest and most pronounced biodegradation was observed with the

powder of the polymer, where it reached more than 80% at the end of the observation period and was comparable with the biodegradability of microcrystalline cellulose powder used as a reference material.

#### B. Biodegradation in soil

Biodegradation of the described forms of polymer was also tested in soil environment. Five agricultural soils were used in the test and their classification types and basic structural and physical-chemical characteristic were determined (Table I). Basic microbiological characteristic were also obtained (Table II) to prove that all five soils were fertile and capable to support biodegradation.

However, the results of biodegradation experiments with all five soils were identical; in no one of the soils any significant biodegradation of any of the polymers forms was observed, levels of mineralization observed was not significantly different from zero.

### IV. DISCUSSION

The polymer proved its good biodegradability under composting conditions. However, our experiments clearly showed that specific surface of polymer specimens affects greatly the rate of biodegradation. For most applications the polymer will be applied in the form of films with a relatively low specific surface and subsequently retarded biodegradation. It must be admitted that the films used throughout our experiments were thicker than films that would be prepared for real applications. We was not capable to prepare films thinner than 100  $\mu\text{m}$  by compression molding, in real industrial

TABLE I  
PHYSICAL-CHEMICAL PROPERTIES OF SOILS

Soil	Horizon	Soil type	Soil texture	pH H <sub>2</sub> O	pH KCl	SOM %
S1	Plough layer	Eutric Cambisol	Loam	7.10	5.18	2.56
S2	Plough layer	Haplic Luvisol	Silty clay loam	6.44	6.33	1.67
S3	Plough layer	Haplic Chernozem	Silt loam	7.28	6.28	3.05
S4	Topsoil	Haplic Fluvisol	Silt loam	7.04	5.49	4.30
S5	Plough layer	Calcic Chernozem	Loam	8.06	7.52	2.07

pH H<sub>2</sub>O = Soil pH in distilled water, pH KCl = Soil pH in M KCl, SOM = Soil organic matter [10].

production the film would be prepared by blowing process and

TABLE II  
BASIC MICROBIOLOGICAL CHARACTERISTIC OF SOILS

Soil	Filamentous fungi	Acrobic chemoorganotrophs	Actinomycetes
S1	$6.89 \times 10^4$	$4.59 \times 10^6$	$3.44 \times 10^6$
S2	$3.78 \times 10^3$	$5.26 \times 10^6$	$2.92 \times 10^6$
S3	$5.37 \times 10^4$	$5.65 \times 10^6$	$3.30 \times 10^6$
S4	$2.25 \times 10^5$	$9.81 \times 10^6$	$6.18 \times 10^6$
S5	$6.65 \times 10^4$	$4.83 \times 10^6$	$4.83 \times 10^6$

Numbers represents colony-forming units (CFU) per gram of soil dry weight.

would be thinner (about 20  $\mu\text{m}$ ).

On the contrary, the polymer revealed to be non biodegradable in soil environment at least under conditions and time scale defined in our experiments. This finding is not completely surprising, because biodegradation of similar materials in soil was not clearly reported in the literature yet. Also longer time studied polyester poly lactid acid (PLA) was shown to be virtually non-biodegradable in soil environment, while readily biodegradable under composting conditions [11,12, 13].

Reasons for such behavior of polyesters are not completely understood. It is known that extracellular lipases and/or peptidases play critical role during depolymerization process and make polymer fragments available for microorganisms. Thus higher ability of compost specific microbial communities to produce such enzymes could be an explanation of faster biodegradation of described polyester in compost environment. This explanation can be supported by the observation of biofilm of bacterial filaments bearing endospores (Fig. 1D) that could be tentatively indentified as thermo-actinomycetes, which is the typical taxonomic group participating on the decay of organic matter in compost.

Not fully rejected there is still the hypothesis that polyesters can be hydrolyzed abiotically or enzymatically and the increased temperature in compost (about 60°C) kinetically accelerates the reaction.

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## Preparation of submicroparticles based on biodegradable co-polyester

Petr Stloukal, Vladimír Sedlárik, Lucie Husarova, Vera Kasparkova, Marck Koutny

**Abstract**— The preparation of submicro particles from biodegradable copolyester by the method of emulsification and solvent evaporation at reduced pressure was investigated. During the study the influence of various process parameters and conditions such as stirring speed, amplitude of sonication and concentration of polymer on the particle diameter and particles size distribution was observed. It was found that it is not possible to achieve a diameter lower than 1  $\mu\text{m}$  without sonication at a relatively high concentration of copolyester. Without sonication the typical mean diameter ranged from 1-10  $\mu\text{m}$ . On the other hand, sonication enabled to prepare particles with diameter lower than 1  $\mu\text{m}$ . A significant influence of the concentration of polymer on final diameter was found, too.

**Keywords**—microparticles, Oil-in-water (O/W) emulsion solvent evaporation method, biodegradable copolymer

### I. INTRODUCTION

THE increasing interest in the area of polymer microparticles is due to a number of promising applications in pharmacology [1], [2], agriculture [3], [4], and environmental engineering. One of such applications is filling polymer microparticles with bioactive a low molecular weight compound which is subsequently, under suitable conditions, released in a controlled way. This application has been extensively studied in recent years. A central issue is the releasing rate of active agents, which is influenced by various parameters, for instance particle diameters or particles size distribution (PSD), as described in [5]. The size of particles and PSD depends on the chosen method of preparation and the process parameters and conditions.

Micro and submicroparticles can be prepared in several ways. However, in the literature, two preparation processes predominate. The first one is the Oil-in-water (O/W) emulsion solvent evaporation method [6], [7]. It is a popular way to get microparticles, which is described in a number of research papers in details and can also be readily realized in the

laboratory without the need for specialized equipment [8]. On the other hand, the second method, which is based on the utilization of supercritical fluids [9], [10], requires more sophisticated laboratory equipment.

Regarding the availability of the O/W method and the lack of sufficient data taking into account the influence of process parameters and conditions on the resulting particles diameter/PSD, the present work is focused on investigation and evaluation of these effects.

In some of research works [2], [3], [5], [7] which are oriented primarily on the release of bioactive compounds, the influence of the concentration of stabilizer in suspension, stirring speed [5], [7] and solvent [7] were observed. However, after closer study of the O/W emulsion solvent evaporation method, it is possible to find that the particles diameter and PSD are influenced by other process parameters and conditions, such as concentration of polymer in solvent, solvent/water ratio in emulsion or type of stabilizer. Particles which were investigated in all of these studies comprised encapsulated bioactive compound. It is presumable that even such low molecular weight compounds can have a certain influence on the size of particles.

In some papers [11]-[13] ultrasonication, which can considerably help to achieve smaller particles in the resulting suspension, was used during preparation. Although it is an indispensable factor in the preparation by the O/W method, the impact of process parameters such as time and amplitude on the resulting diameter and PSD has not been satisfactorily investigated and described yet.

In this study the possibilities of preparation of micro and submicroparticles from commercially available biodegradable copolyester were tested. The influence of stirring speed of the homogenizer and sonication amplitude on the particle diameters and PSD is evaluated and compared with the literature.

### II. MATERIALS AND METHODS

#### A. Materials

Polymer used throughout the study was aromatic-aliphatic copolyester Ecoflex purchased from BASF (Germany). Airvol 205 - polyvinylalcohol (JVP, Japan) was applied as an emulsion stabilizer in the form of 0.5 % aqueous solution.

#### B. Preparation of particles

Micro and submicroparticles were prepared by the

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P. Stloukal, M. Koutny and L. Husarova are with Department of Environmental Protection Engineering, Faculty of Technology, Tomas Bata University in Zlín, 762 72 Zlín, Czech Republic (corresponding author, phone: 00420-604-559-681; e-mail: mkoutny@ft.utb.cz).

V. Sedlárik is with Polymer Centre Faculty of Technology, Tomas Bata University in Zlín, 762 72 Zlín, Czech Republic.

V. Kasparkova is with Department of lipids, tensides and cosmetics, Faculty of Technology, Tomas Bata University in Zlín, 762 72 Zlín, Czech Republic.

oil-in-water emulsion solvent evaporation technique. This method was carried out in the following way.

Firstly, the polymer (2, 1.5, 1, 0.5, 0.25 g) was dissolved in 10 ml of chloroform. The resulting concentration of polymer in chloroform (P/C concentration) was 200, 150, 100, 50, 25 mg ml<sup>-1</sup>. Chloroform was primarily used because it has the higher boiling point in comparison with similar solvents such as dichloromethane. The relatively high value of boiling point ensures that chloroform will not evaporate from the mixture when the emulsion is prepared by vigorous stirring.

Then, the polymer solution was emulsified into 0.5 % (w/v) water solution of PVA (emulsifying agent) and dispersed under the continuous stirring by the homogenizer Ultraturax (IKA T18, Germany) at stirring speeds of 6000, 10000, 14000, 18000, 22000 and 24000 rpm. The mixture was stirred for 10 min. and during this period the produced emulsion was cooled with ice. The ratio between water solution of PVA and chloroform solution of Ecoflex was the same in all experiments (4:1).

Individual series of samples were marked S, U, A, C. For samples S ultrasonication was not used in second step of the procedure.

For labelings U, A, C, the obtained emulsion was ultrasonicated by ultrasonic probe (Hielscher UP 400S, Germany) for 5 min, when the cycle of sonication was 0.5 and amplitude 20, 25, 30, 35, 50, 70 %.

At last, the prepared emulsion (for all labels) was placed

into the erlenmayer flask and stirred. Subsequently the organic solvent was evaporated from the emulsion under reduced pressure and the suspension of microparticles was formed. Reduced pressure was created by a membrane pump. The samples were stored in freezer at -20 °C for analyses.

### C. Particle characterization

Particle parameters were measured with the help of optical microscope. During the microscopic investigation, the samples were placed on a glass slide with a graduated grid.

Where the diameter of particles allowed (particles smaller than 5 µm), the particle distribution was measured with Zetasizer (Zetasizer Nano ZS, Malvern Instruments, UK).

### D. Evaluation of images from the optical microscopy

The length of the edge of a square in graduated field was 50 µm. From each image, six squares were randomly chosen for evaluation. The number of particles was counted in each square for all predetermined size intervals.

## III. RESULTS AND DISCUSSION

Table I shows the conditions used to prepare the micro and submicroparticles and the obtained mean diameter (weighted average relative to the intensity of scattering). As can be seen, except for samples of S1-S6, the mean diameter of particles below 1 µm was achieved due to sonication.

In comparison with literature [5]-[7], where preparation was

TABLE I  
THE CONDITIONS USED TO PREPARE THE MICRO AND SUBMICROPARTICLES AND THE OBTAINED DIAMETER

Sample	Stirring speed (rpm)	Polymer concentration (mg ml <sup>-1</sup> )	Ultrasonication	Amplitude (%)	Mean diameter of particles (µm)
S1	6000	100	no	-	9.44
S2	10000	100	no	-	4.74
S3	14000	100	no	-	2.60
S4	18000	100	no	-	2.64
S5	22000	100	no	-	1.94
S6	24000	100	no	-	1.74
U1	6000	100	yes	35	not determined
U2	10000	100	yes	35	0.94
U3	14000	100	yes	35	0.79
U4	18000	100	yes	35	0.94
U5	22000	100	yes	35	0.87
U6	24000	100	yes	35	0.57
A1	24000	100	yes	20	0.88
A2	24000	100	yes	25	0.79
A3	24000	100	yes	30	0.89
A4	24000	100	yes	35	0.57
A5	24000	100	yes	50	0.59
A6	24000	100	yes	70	0.72
C1	24000	25	yes	35	0.40
C2	24000	50	yes	35	0.61
C3	24000	100	yes	35	0.57
C4	24000	150	yes	35	0.95



performed without sonication, the diameter of the obtained particles was always in order of micrometers, whereas in research papers [12]-[14], where sonication was used, particles smaller than 1  $\mu\text{m}$  were achieved.

The results are discussed in details in further paragraphs. It is necessary to say that the comparison with the mentioned research papers has some limitations, which will be discussed, too.

#### A. Influence of stirring speed on particle distribution

The mean diameters of particles for samples S1-S6 (described in Table 1) are shown in Figure 1.

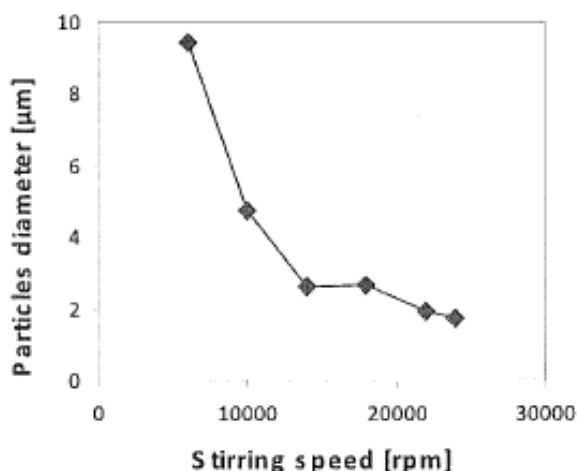


Fig. 1 Dependence of the mean particle size on stirring speed without ultrasonication

As can be seen, whereas in the interval from 6000 rpm to 14000 rpm the particle size is strongly affected by the stirring speed, for speeds over 14000 rpm the medium particle size remains almost constant. The application of higher stirring speed than 14000 rpm is not very efficient for the further reduction of mean diameter. As mentioned before, with stirring only we did not succeed to prepare particles under diameter 1  $\mu\text{m}$ . In samples which were stirred at speed 14000 rpm and higher, the largest particles were reduced and mean diameter over 3  $\mu\text{m}$  was achieved. However, the certain number of particles was bigger than 5  $\mu\text{m}$ , therefore z-sizer could not be used to measure their diameters.

In the research work [6], where the same method and similar process conditions were used, the diameter of particles in the order of micrometer was also obtained. The authors observed polymer with the molecular weight higher than 100,000, which corresponds with ours (polymer molecular weight higher than 100,000).

It is presumable that the achievement of the diameter under 1  $\mu\text{m}$ , when the similar polymer (molecular weight higher than 100,000) and process conditions are used, is impossible without sonication. However, the particles with the diameter lower than 1  $\mu\text{m}$  could be successfully obtained if some process parameters, such as initial concentration of polymer in the organic solvent, are changed. Unfortunately, there is a lack

of information from the literature, to prove our presumptions.

The particles diameter obtained with each stirring speed was calculated from the data in Figure 2. In this figure, the comparison of particles size distributions for different stirring speeds is presented. The overall shift and the decrease of particle size distributions with the increasing stirring can be observed. The larger particles disappear gradually and, practically, only smaller ones remain. The shift of PSD to the lower values of particle diameters can be probably achieved by using longer time of stirring. However, the mentioned assumption should be verified by further investigation.

Figure 3 shows the significant difference between particles prepared by the lowest stirring speed (6000 rpm) and the highest speed (24000 rpm). The diameter of all particles was approximately determined with the help of calibrated grid, which is perceptible in the figure.

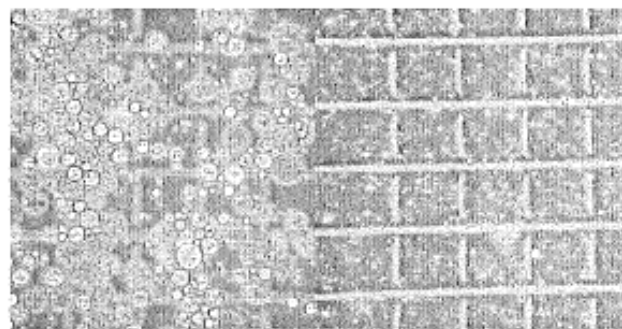


Fig. 3. Particles prepared at 6000 rpm (left) and 24000 rpm (right) the size of calibration chamber squares is 50  $\mu\text{m}$

As can be seen, the number of particles increased and size decreased with higher of stirring speed. This trend results in the inaccuracies of the measurement of the particle diameter and subsequent calculations for higher stirring speeds.

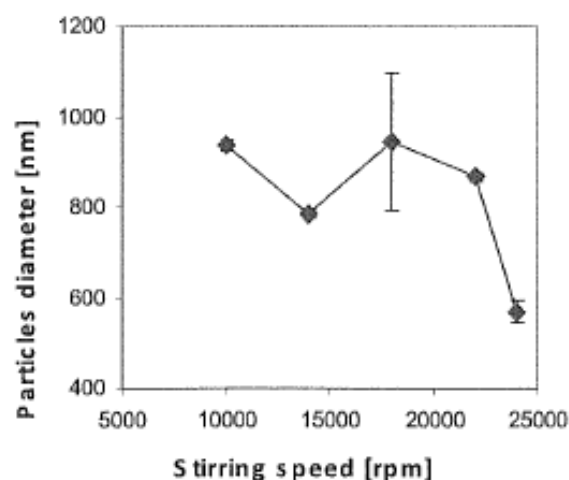


Fig. 4 Dependence of the mean particle size on stirring speed with ultrasonication, points are averages from 3 measurements. Error bars represent twice standard deviations.

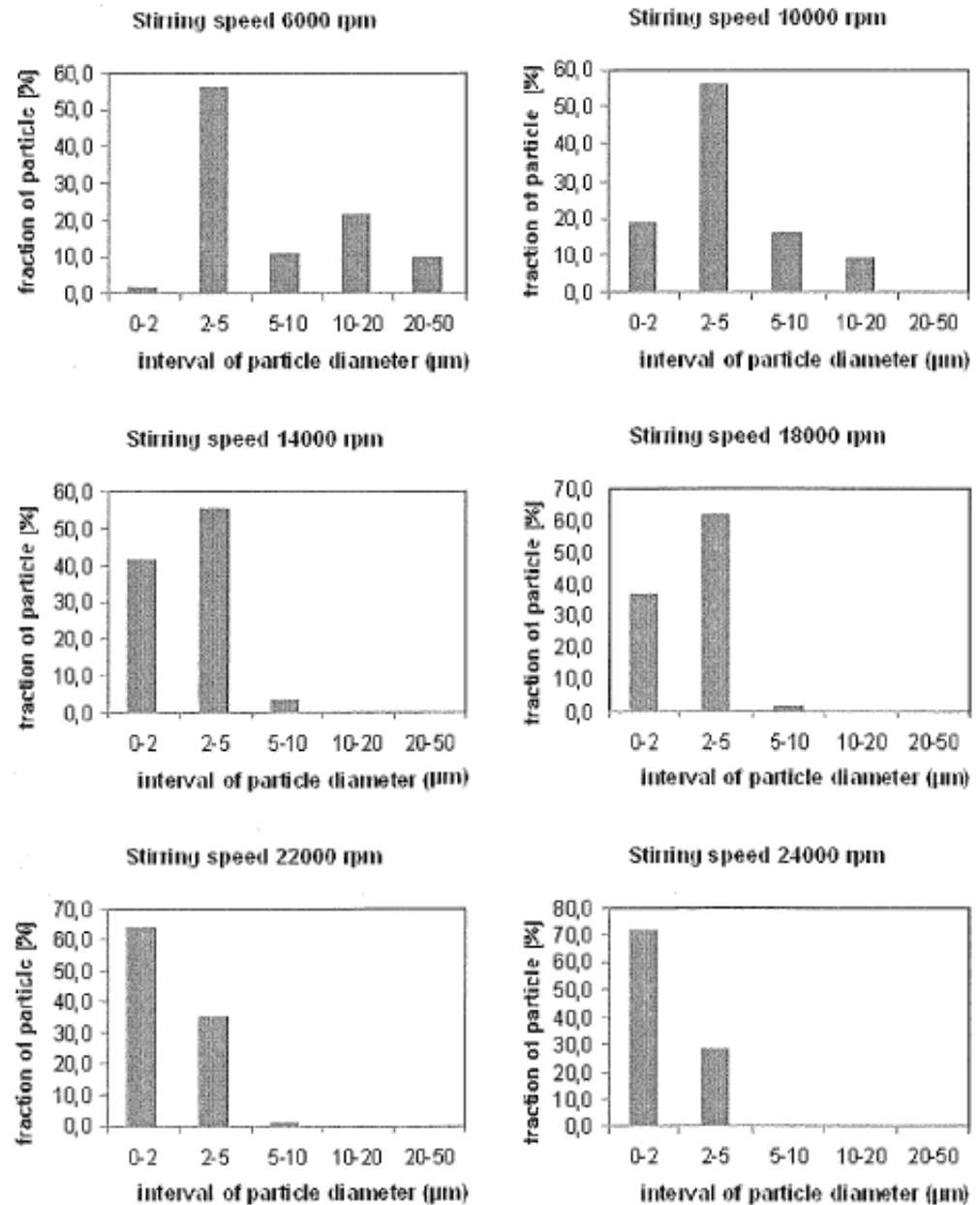


Fig. 2 Comparison of particles size distributions for different stirring speeds.

Figure 4 shows the dependence of particles diameters on stirring speeds. In this case ultrasonication was used during the preparation. The process parameters for samples U1- U6 are described in Table 1.

The mean particle diameter was reduced under 1 µm. After ultrasonication, PSD were shifted significantly toward lower diameters, which made possible to use z-sizer for their characterization. As can be seen in figure 4, the measured values of particles diameter for stirring speed 6000 rpm were not used because they seem to be unreliable. The wider

standard deviation of particle diameter for stirring speed 18000 rpm is probably caused by non-homogeneity of the sample. It is evident that the particle diameter after ultrasonication is not so strongly dependent on the stirring speed. For almost all stirring speeds the particles diameters are very similar. However, for the highest homogenization stirring speed (24000 rpm) significantly smaller particles were obtained.

In literature [12]-[14], much smaller particle diameter was achieved. Main reason seems to be the use of considerably



lower concentrations of polymers, which had, together with amount of stabilizer in suspension, important influence on the final mean diameters and PSD.

In [13] lower particle diameter was achieved even with electromagnetic stirring only (stirring speed 500 rpm). It is possible that ultrasonication has bigger influences on particles diameter and PSD than stirring speed. However, in all of these research papers the polymers with the molecular weight lower than 100,000 were used in contrast with Ecoflex which has the molecular weight higher than 100,000.

#### B. Optimization of ultrasonication procedure

Figure 5 presents the dependence of particle size on the amplitude of ultrasonication for the series of samples A1 – A6. As can be seen, the amplitude was in the range of 20 % to 70 %. A sharp critical value close to 35% amplitude is apparent from the figure. It is very interesting that larger particles are formed for the amplitude 70 % than for the 35 %.

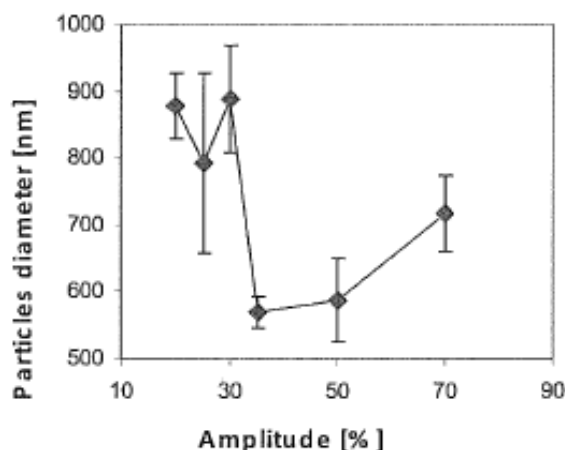


Fig. 5 Dependence of mean particle size on the amplitude of ultrasonication parameter. Points are averages from 3 measurements. Error bars represent twice standard deviations.

It could be explained by the partial agglomeration of particles instead of their reduction, which is caused by the great amount of energy delivered into the emulsion and possibly the local increase of temperature using higher amplitudes.

#### C. Influence of the polymer concentration

Finally, the measurement was performed for the series of samples C1-C5. The influence of polymer concentration was investigated.

As can be seen from Figure 6, the particle diameter increased rising the concentrations. The trend of increasing particle diameters with rising concentrations was also detected in [12] and [14] and could be explained in various ways, for example too small volume of the water phase, too short time of stirring and ultrasonication for higher concentrations of the

polymer. The concentration of emulsion stabilizer (PVA) in emulsions has probably a crucial influence. For a large quantity of Ecoflex in the emulsion, the amount of stabilizer is unable to cover the surface of all of the small particles formed.

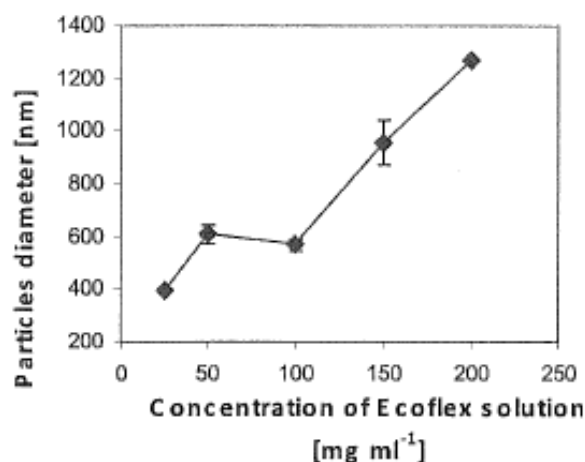


Fig. 6 Dependence of the mean particle size on the concentration of Ecoflex solution. Points are means from 3 measurements. Error bars represents twice standard deviations.

In the paper [14] authors presumed that the enlargement of nanoparticles is probably caused by the increasing viscosity of dispersed phase (more concentrated polymer solution), resulting of a poorer dispersability of the polymer solution into the aqueous phase caused by a high viscous resistance against the shear forces during emulsification. From which it follows that it is possible to obtain smaller particles from more concentrated polymer solutions by using either longer time of stirring or ultrasonication or both.

In our case, the linear dependence of the particle diameter on the concentration of Ecoflex solution can be observed. In [14], where three different but rather low concentrations were investigated the values of particles diameters for the two lowest concentrations were almost identical. It is possible, that lower diameters are not achieved by further reduction of the concentration under some limit.

#### IV. CONCLUSION

The study has proved that it is possible to prepare micro and submicro polymer particles in the procedure comprising emulsification and organic solvent evaporation at reduced pressure. Among the investigated processing parameters, sonication was found to be the crucial step in particle preparation that makes possible to prepare particles of diameters under 1  $\mu\text{m}$ .

Further research should be focused on the investigation of the influence of other process parameters, namely the nature of the emulsifying agent and concentrations of all components used throughout the procedure and their influence on the particle size.

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## Příloha P8: Influence of long term storage on thermooxidation behavior of prooxidant containing low density polyethylene (plnotextový příspěvek na konferenci)

Mathematical Models and Methods in Modern Science

### Influence of long term storage on thermooxidation behavior of prooxidant containing low density polyethylene

L.HUSAROVA<sup>1,2</sup>, M. KOUTNY<sup>1,2</sup>, P. STLOUKAL<sup>2</sup>

<sup>1</sup>Centre of Polymer Systems, Tomas Bata University in Zlin, nam. T.G.Masaryka 5555, 760 01 Zlin

<sup>2</sup>Environmental Protection Engineering, Faculty of Technology, Tomas Bata University in Zlin, nam. T.G.Masaryka 5555, 760 01 Zlin  
mkoutny@ft.utb.cz

*Abstract:* - Thermooxidation curves of two low density polyethylene films containing prooxidant additives were compared between original fresh samples and the same samples stored three years in refrigerator at 4°C. It was observed that both investigated films changed its behavior dramatically and after the storage exhibited depletion of antioxidant capacity with was manifested mainly as decrease or complete disappearance of the initial induction period. Such phenomenon could have important consequences on the applicability of this type of materials.

*Key-Words:* - polyethylene, prooxidants, thermooxidation, biodegradation, FTIR

#### 1 Introduction

Many recent studies have focused on polyethylene (PE) as a material which is widely employed especially due to its low cost. This is the reason why we are surrounded by it everywhere. PE is utilized on a massive scale as packaging material, films (as mulching films in agriculture, stretch films in grocery), shopping bags, sacks etc. Moreover, products from PE are single-used which is in contrary with PE's long lifetime [1, 2].

Thought PE is non-toxic it is impossible to look over the fact that discarded packaging is responsible for the death of a large number of mammals by ingestion and strangulation [3].

Because PE is material with an extremely high molecular weight his resistivity in the environment is increased. Chains are assembled from uniform –CH<sub>2</sub>- units, hence, the polymer is hydrophobic and bioinert for microorganisms (MO), i. e. PE can not be assimilated by MO [4]. Separation and recycling of PE is often economically and energetically expensive, therefore new methods of degradation are searched and examined with biodegradation being one of the most promising.

Majority of PE products contain stabilizers which prevent their oxidation not only during the processing but also at subsequent decomposition. The degradation process can be accelerated by addition of compounds called prooxidants, which support oxidation of the polymer chains after

The basic principle of prooxidants function is the incorporation of transition of metal ions (Mn<sup>2+</sup>, Co<sup>2+</sup>, Fe<sup>3+</sup>) [5] that induce accelerated oxidation in the material. Also with participation of UV radiation and/or heat the polymer is fragmented, chains of PE become shorter, contain more polar groups and are more available for MO [6].

In our previous papers [7, 8] we investigated PE with prooxidants and showed that thermooxidation of the material is characteristic by two distinct phases. First, when introduced antioxidant capacity is consumed and the material retains its mechanical and spectroscopic properties and second beginning after the consumption of prooxidants when material is rapidly oxidized, loosing its mechanical properties. This process can be easily monitored by FTIR spectroscopy and accelerated by increase of temperature. One of the conclusions from these studies was that the process does not have simple linear Arrhenius temperature dependence.

Here we present study where we repeated the previously described experiments after the material was stored for three years at 4°C. In theory and considering only the linear Arrhenius temperature dependence we should observe almost no change comparing to three years old experiments. We also expanded our observations to lower temperatures.

## 2 Materials and methods

### 2.1 Tested material

Two materials were studied LDPE-1, low density polyethylene film 50-55  $\mu\text{m}$  thick and which contained 15% of micro-milled lime as filler and 5% of commercial prooxidant additives (Addiflex HE). The additive is based on manganese ions and LDPE-2, low density polyethylene film 30-35  $\mu\text{m}$  thick films containing prooxidants based on manganese and iron ions.

## 3 Results and discussion

Two distinct phases of the material oxidation can be clearly seen on the curves obtained with fresh material relatively short time after it was processed. First, initial lag phase or induction phase, when antioxidants present in the material are consumed, and then a very fast oxidation phase that follows. These data were previously published and commented [7, 8]. After three years of storage we expected having qualitatively the same curves with important increase of lag phase for 50°C incubation.

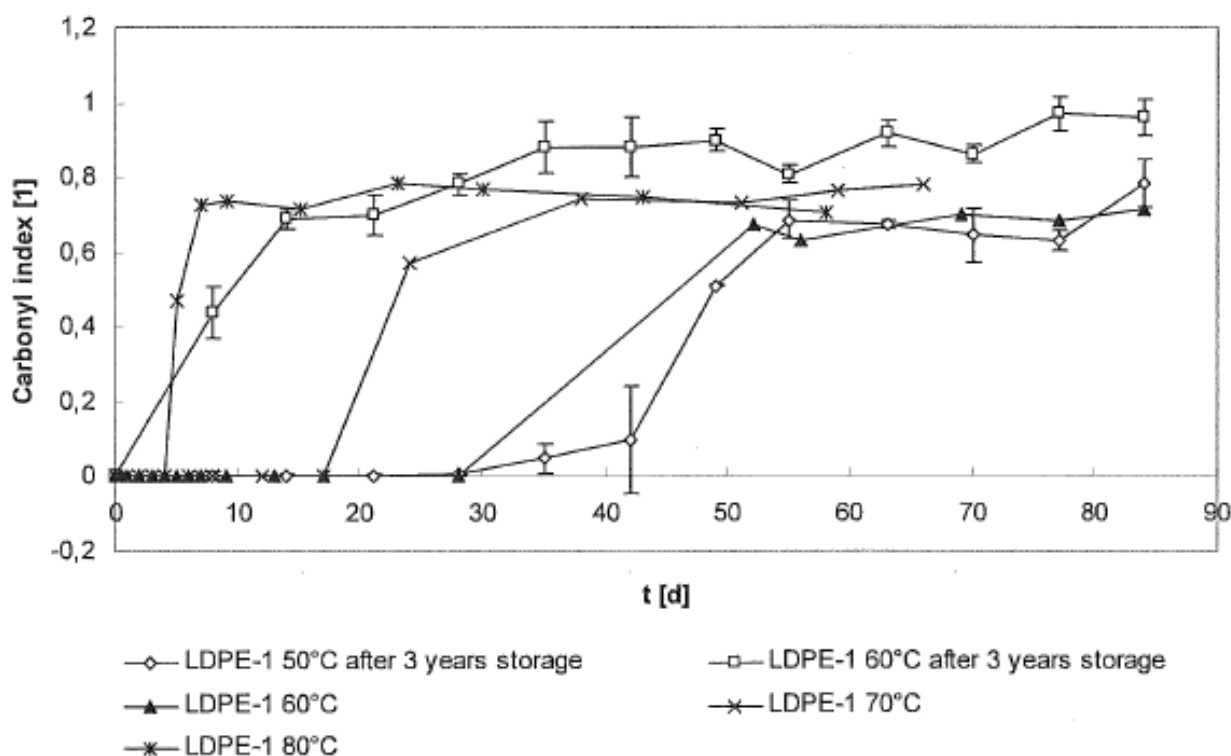


Fig.1. Carbonyl index evolution of LDPE-1 samples thermooxidated at different temperatures. Fresh samples and samples stored three years at 4°C were compared.

### 2.2. FTIR

Both samples were cut into regular rectangular flakes 3 cm long and 2 cm wide and stored at 4°C for three years then the samples were incubated at 50 and 60°C on air for approximately 90 days, subsamples were withdraw in regular intervals and characterized.

Structural changes occurring in these materials were investigated using FTIR spectroscopy. The FTIR spectra were recorded at regular intervals using a Mattson 3000 (UNICAM, UK) spectrophotometer [9]. The carbonyl index (CI) was calculated as the ratio of absorbance values at 1713 and 1465  $\text{cm}^{-1}$ .

In fact it was broadly supposed that at 4°C the consumption of antioxidants was so slow that it could be regarded as insignificant. Contrary to this expectation we saw dramatic differences in the characters of the curves and in the lengths of the described induction phase.

In the case of LDPE -1 (Fig. 1), the induction phase in 60°C incubated samples disappeared completely or was so unexpectedly short that could not be noticed. The curve for 50°C was parallel with the 60°C curve obtained three years ago. LDPE-2 sample altered its behavior even more dramatically when comparing with previously reported results (Fig. 2).

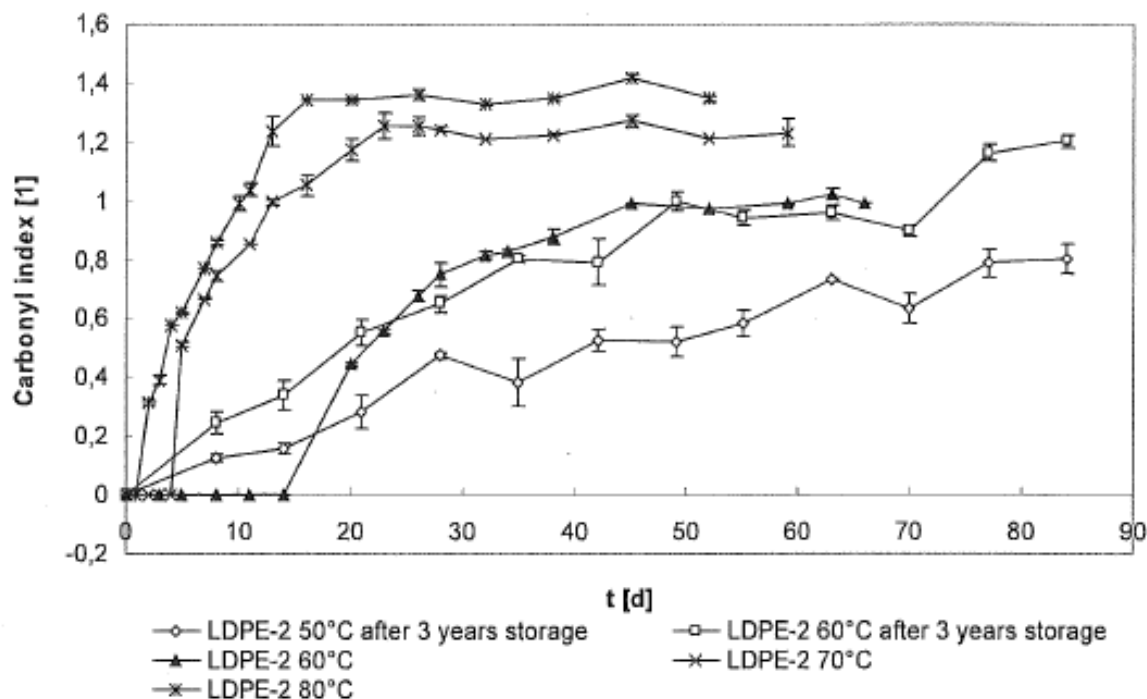


Fig.2. Carbonyl index evolution of LDPE-2 samples thermooxidated at different temperatures. Fresh samples and samples stored three years at 4°C were compared.

For both temperatures induction phases disappeared completely, but also the oxidation phase was altered and was slower and almost linear which is contrast with previously observed very steep and short oxidation period followed with a plateau.

The observed changes are quite complex and cannot be explained in a sufficiently straightforward way but it is evident that important part of antioxidation capacity was consumed in LDPE-1 and that it was depleted probably completely in LDPE-2 during the storage period.

#### 4 Conclusion

Contrary to the expectations and general believe it was found that during the storage even at relatively low temperature 4°C the balance between prooxidant and antioxidant activity in the material was dramatically changed. Antioxidant activity decreased considerably so the whole idea of preprogrammed properties of the polyethylene with prooxidant additives could prove to be insufficiently robust.

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