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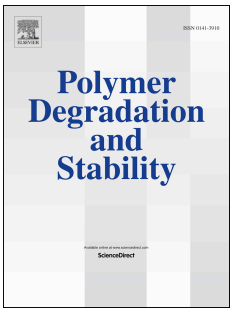


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# Biodegradation of poly(ε-caprolactone) (PCL) and medium chain length polyhydroxyalkanoate (mcl-PHA) using whole cells and cell free protein preparations of *Pseudomonas* and *Streptomyces* strains grown on waste cooking oil

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Running title: PCL and mcl-PHA degradation with bacteria grown on waste cooking oil

Abbreviation list

**ATR-FTIR** *Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy*

**PHA** *polyhydroxyalkanoates*

**scl-PHA** *short-chain length polyhydroxyalkanoates*

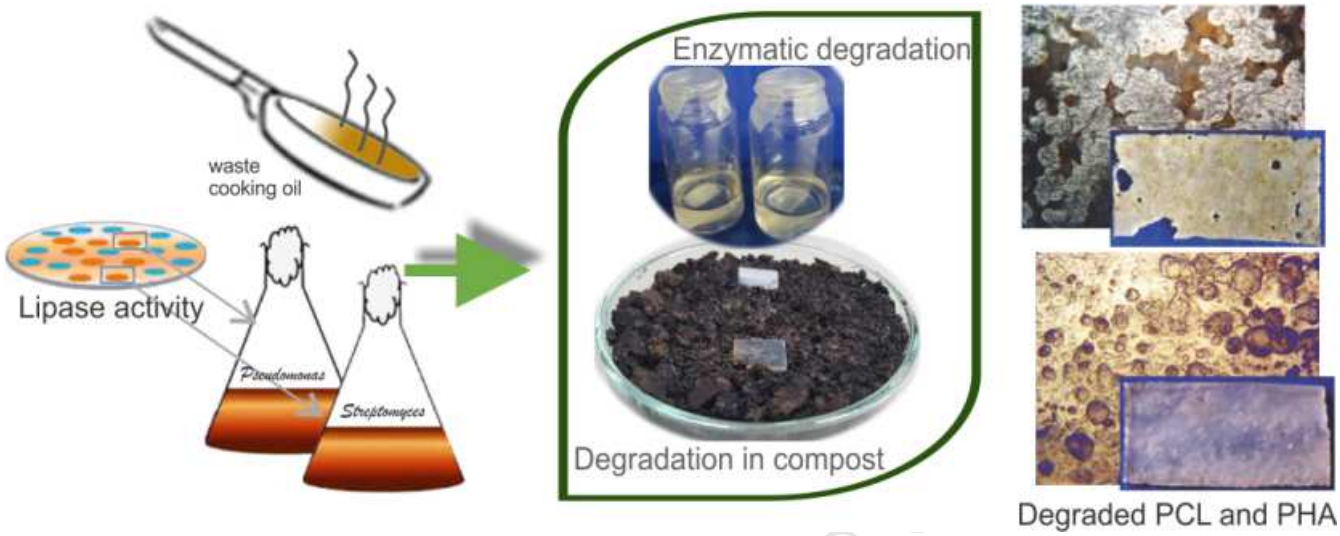
**mcl-PHA** *medium-chain length polyhydroxyalkanoates*

**lcl-PHA** *long-chain length polyhydroxyalkanoates*

**PHB** *polyhydroxybutyrate*

**PCL** *poly(ε-caprolactone)*

42 **Graphical abstract**

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**Abstract**

Petrochemical plastics are generally recalcitrant to microbial degradation and accumulate in the environment. Biodegradable polymers obtained synthetically like poly(ε-caprolactone) (PCL) or polyhydroxyalkanoates (PHA**)**, obtained biotechnologically, have shown great potential as a replacement for petroleum-based plastics. Nevertheless, their biodegradation and environmental faith have been less examined. In this study, thin films of PCL (200 µm) and medium chain length PHA (mcl-PHA, 70 molar fraction of 3-hydroxyoctanoate and 30 molar fraction of 3-hydroxydecanoate, 600 µm) were exposed to total protein preparations (extracellular proteins combined with a crude cell extract) of soil isolates *Pseudomonas chlororaphis* B-561 and *Streptomyces* sp. BV315 that had been grown on waste cooking oil as a sole carbon source. Biodegradation potential of two polyesters was evaluated in buffer with total protein preparations and in a laboratory compost model system augmented with selected bacteria. Overall, PCL showed better biodegradation properties in comparison to mcl-PHA. Both materials showed surface erosion after 4-weeks of exposure to total protein preparations of both strains, with a moderate weight loss of 1.3% when *P. chlororaphis* B-561 was utilized. In laboratory compost model system PCL and mcl-PHA showed significant weight loss ranging from 13 to 17% when *Streptomyces* sp. BV315 culture was used. Similar weight loss of PCL and mcl-PHA was achieved for 4 and 8 weeks, respectively indicating slower degradation of mcl-PHA. Growth on waste cooking oil as a sole carbon source increased the potential of both tested strains to degrade PCL and mcl-PHA, making them good candidates for augmentation of compost cultures in waste management of both waste cooking oils and biodegradable polymers.

**Keywords** biopolymers, enzymes, *Pseudomonas*, *Streptomyces*, biodegradation, compost

## 1. Introduction

With the advances in technology and the increase in the global population, petroleum-based plastics have replaced traditional materials such as metal, leather, and wood because of their mechanical strength, lightness, flexibility and versatility (Sivan, 2011). The most desired characteristic of the plastic material, durability, and resistance to different environmental factors, also presents major hazards for the environment (Sivan, 2011, Prieto, 2016). Plastic waste which accumulates at a staggering rate of 28 million tons per year combined with poor post-consumer management has created the persistent contamination of water and soil thus making it one of the major sources of environmental pollution (Avérous & Pollet, 2012, Bhardwaj *et al.*, 2013). Microbial decomposition of plastic waste is estimated to be low, even though some bacterial species, like *Streptomyces*, are capable of colonizing and degrading plastics (Li *et al*., 2016). This phenomenon is driving the development of polymers with biodegradable properties for applications in packaging, agriculture, and single-use items such as cutlery (Banerjee *et al.*, 2014, Albuquerque & Malafaia, 2018). There are two types of biodegradable polymers: petroleum-based polymers that can be degraded by microorganisms including poly(ε-caprolactone) (PCL) and poly(butylene succinate) (PBS) and bio-based polymers derived from biomass or renewable resources such as polyhydroxyalkanoates (PHA), polylactic acid (PLA) and thermoplastic starch (TPS), that can also be degraded by microorganisms (Tokiwa *et al.*, 2009, Wierckx *et al*., 2018). For a long time, it was assumed that biopolymers produced by microorganisms as a part of their metabolic pathways are readily biodegraded. However, addressing waste management of bio-based polymers is required, as it has been shown that these polymers and their blends are not always biodegradable or may require conditions only available in industrial-scale composting facilities (Narancic *et al*., 2018, Prieto, 2016, Hottle *et al.*, 2017).

PCL, a biodegradable polyester, synthesized from a petroleum-based monomer, has a low melting ($60^{O}$C to $65^{O}$C) and glass transition temperature ( - $65^{O}$C to - $60^{o}$C), good water, and oil resistance, is easily soluble most often used solvents and is easily processed from the melt (Woodruff, 2010). It has been shown that it can be degraded by the action of widely distributed aerobic and anaerobic microorganisms in various ecosystems including soil (Tokiwa *et al.*, 2009). On the other hand, PHAs are entirely a product of microbial metabolism, produced in environments with limited essential nutrients and usually intracellularly accumulated as carbon and energy storage in the form of granules (Prieto, 2016, Narancic & O'Connor, 2017). At present, PHAs are classified in three major classes: short-chain-length PHAs (scl-PHAs) containing C3-C5 carbon atoms in monomers, medium-chain-length PHAs (mcl-PHAs) with C6-C14 monomers and long-chain length PHAs (lcl-PHA) containing monomers with more than 14 carbon atoms which reflects in their material properties such as crystallinity and thermal properties. As biocompatible and biodegradable polyesters composed of hydroxylated alkanoic acids, that can be directly produced by a wide range of microbes from renewable resources, PHAs have emerged as a potential alternative to synthetic polymers as they are preferable from the perspective of human health and environment safety (Gross & Kalra, 2002, Alvarez-Chavez *et al.*, 2012, Albuquerque & Malafaia, 2018).

The ability of microorganisms to enzymatically degrade polymers into low molecular weight oligomers, dimers and monomers may be used as a powerful tool for treatment and recycling of biodegradable wastes (Tokiwa *et al.*, 2009). As previous studies showed, different bacterial and fungal species have the ability to degrade biodegradable polymers. Among the microbial population, species of *Pseudomonas*, *Streptomyces*, *Rhodococcus*, *Comamonas*, *Clostridium*, and *Butyrivibrio* have been shown to be the dominant bacterial species that have the ability to degrade polymers (Pathak & Navneet, 2017). For the

degradation of large molecules which cannot enter the microbial cell, secretion of specific enzymes that hydrolyze the constituents of biodegradable polymers is essential. These microbial exoenzymes historically have been involved in the carbon cycle and their expression is usually inducible (Arnosti, 2011, Juturu & Wu, 2012). Among the important enzymes are lipases and other esterases, as well as PHA depolymerases, if PHAs are specifically targeted because they accelerate the degradation of polymers by attacking the polymer backbone and producing oligomers (Pastorino *et al.*, 2004, Azevedo & Reis, 2005, Knoll *et al.*, 2009).

In the growing demands for sustainable waste management treatments, *Pseudomonas* and *Streptomyces* strains which possess the high activity of biopolymer degrading enzymes (Jaeger & Rosenau, 2004, Spasic *et al.*, 2018) are showing a great potential for biodegradation of PCL (Chua *et al.*, 2013, Ponjavic *et al.*, 2017a), as well as scl-PHAs and mcl-PHAs (Santos *et al.*, 2013, Martinez *et al.*, 2015) under laboratory conditions. Such bacteria could be added to managed environments where accelerated biodegradation is required e.g. home composting where many biodegradable polymers do not degrade fast enough to meet international regulatory standards. In addition, the ability of these strains to use waste cooking oil as a sole carbon source for their growth and induction of polymer-degrading enzymes would make the biodegradation an added-value process, since the bacteria could be grown on waste stream for the degradation of other type of wastes. The existing data on biodegradation or composting of mcl-PHAs are very limited and there are no studies that connect the use of waste oil for microbial growth and polymer biodegradation or composting. It was shown that lipases could degrade polymers (Palanisamy *et al.*, 2016; Ponjavic *et al*., 2017a), and it is also known that synergism with other polymer-degrading enzymes such as cutinases and PETases makes degradation process more efficient (Carniel *et al*., 2017). Thus the aim of this study was to assess the potential of selected non-pathogenic *Pseudomonas* and *Streptomyces* soil isolates to

grow on waste oil stimulating the expression of esterase (lipase)-like enzymes for the purpose of enhanced biodegradation of biodegradable polymers PCL and mcl-PHA materials.

## 2. Materials and methods

### 2.1. Reagents

Rhodamine B and *p*-nitrophenylpalmitate (p-NPP) were purchased from Sigma (St. Louis, USA). Glucose, mannitol, tryptone, peptone, yeast extract and other media components were purchased either from Oxoid (Cambridge, UK) or Becton–Dickinson (Sparks, USA). Solvents, such as acetone, ethanol, and chloroform were of high purity grade and purchased from Sigma (St. Louis, USA).

### 2.2. Preparation of PCL polymer films

PCL films used for degradation experiments were prepared from previously synthesized polyester by ring-opening polymerization in bulk, in the presence of alcohol (ethanol) in order to control molecular weight, and tin(II) octoate as a catalyst following previously described method (Ponjavic *et al.*, 2016). The obtained PCL was of low molecular weight (according to NMR measurements number average molecular weight was 11000 g $mol^{-1}$).

The PCL films were prepared from a 6% polymer solution in chloroform (1.5 g of the polymer sample was dissolved in 25 mL of chloroform) by the solvent casting method in glass Petri dishes (diameter 10 cm) by leaving the solvent to evaporate 12 h at room temperature. After solvent evaporation, polymer films were dried in a vacuum oven for 24 h at room temperature and cut into the rectangles (10 × 20 mm, thickness 200 μm, weight about 40 mg) for the degradation experiments.

### 2.3. Preparation of mcl-PHA polymer films

The mcl-PHA utilised in this study contained 70 molar fraction of 3-hydroxyoctanoate and 30 molar fraction of 3-hydroxydecanoate which are the typical monomers present in mcl-

PHA produced by *Pseudomonas putida* KT2440 from fed-batch fermentation by the method of Davis *et al*. (Davis *et al*., 2015) using glucose and octanoate as carbon source with Mw of 132,000. The mcl-PHA films were prepared in the glass Petri dishes (diameter 10 cm) by solvent casting method from a 20% polymer solution in acetone (15 g of the mcl-PHA polymer obtained from Bioplastech Ltd (Dublin, Ireland) was dissolved in 75 mL of acetone) leaving the solvent to evaporate for 7 days at room temperature. For the degradation experiments obtained films were cut into rectangles (10 × 20 mm, thickness 600 μm, the average mass of 200 mg).

### 2.4. Media for the growth of *Pseudomonas* and *Streptomyces* species

*Pseudomonas* spp. and *Streptomyces* spp. strains used in this study were from the Laboratory for Microbial Molecular Genetics and Ecology (Institute of Molecular Genetics and Genetic Engineering, Belgrade, Serbia) which is a collection of microorganisms isolated from various natural habitats (predominantly soil).

Mineral Salt Medium (MSM) (Schlegel *et al*., 1961) and lysogeny broth (LB) (Bertani, 1951) liquid and solid medium were used for cultivation of *Pseudomonas* species. Mannitol soy flour medium (MSF) and R2 (Kieser *et al*., 2000) were used for the growth of *Streptomyces* species.

### 2.5. The growth of bacterial cultures

*Pseudomonas* spp. strains and *Streptomyces* spp. spore suspensions were stored in glycerol (20%, v/v), maintained at – 80°C and used for the inoculation of cultures. At the time of usage, bacterial cells, as well as spore suspensions (20 μL each) were inoculated onto solid LB agar medium (*Pseudomonas*) and MSF medium (*Streptomyces*) and grown at 30°C overnight and for 7 days, respectively. For degradation experiments, *Pseudomonas* chlororaphis B-561 and *Streptomyces* sp. BV315 strains were grown in liquid MSM and R2 media, respectively, with 0.1% (w/v) glucose and 1% waste cooking oil (v/v). *P. chlororaphis*

B-561 was grown for 72 h at 30°C with shaking at 180 rpm, while *Streptomyces* sp. BV315 strain was grown for 96 h at 30°C with shaking at 200 rpm.

### 2.6. Preparation of total proteins for polymer degradation experiments

Bacterial strains were grown as described in section 2.5. In order to investigate the enzymatic degradation of polymers in the buffer, total protein preparation containing proteins exported to culture supernatants as well as cell-free extracts were prepared. Cells were harvested by centrifugation for 10 min at 4230 × g at 4°C (Sorvall GS3, RC-5B Super Speed Centrifuge; Du Pont Instruments, USA). Supernatants were kept on ice and cell pellets were resuspended in 20 mM sodium phosphate buffer pH 7.4 at 1 g of wet cell weight (1% w/v). Cells were disrupted by sonication using Soniprep 150 sonicator (MSE, UK) with 5 short bursts of 20 s, followed by an interval of 20 s of cooling. Pellets were removed by centrifugation for 40 min at 20817 × g at 4°C (Eppendorf Centrifuge 5417 R, Germany). For each culture, supernatant and cell-free extract were combined to form a suspension of total cell proteins (total protein preparations) in order to analyze polymer degradation.

Total protein concentration in the supernatant and cell-free extract suspensions were determined using coloring reagent CBB G-250 (BioRad Protein Assay, BioRad Laboratories, USA) according to Bradford method (Bradford, 1976). Total protein preparations of both strains were prepared to have the same protein content.

### 2.7. Lipase (esterase) activity

In order to determine lipase activity of strains, total protein preparations ~~o~~f selected *Pseudomonas* spp. and *Streptomyces* spp. strains were tested using plate enzyme assay and colorimetric assay with *p*-nitrophenylpalmitate (p-NPP) as a substrate. Plate enzyme assay was conducted using 1% agarose (w/v) supplemented with 1% waste cooking oil (v/v) and 2 mg $L^{-1}$ of rhodamine B in phosphate buffer. Lipase activity was detected under the UV light as the formation of an orange-colored fluorescent halo (Ugur & Sarac, 2014). For the

quantitative enzyme assay selected strains were grown both on 0.1% (w/v) glucose and 1% waste oil (v/v) as carbon sources and assay was performed according to Pinsirodom and Parkin (Pinsirodom & Parkin, 2001) in 3 mL reaction volume. Reactions were incubated at 37°C with shaking at 180 rpm and absorbance was measured after 10, 20, 30, 60 and 120 min at 410 nm.

**2.8. Polymer degradation on agar plates**

Degradability on agar plates was analyzed for PCL and mcl-PHA polymers. For the purpose of this experiment, *P. chlororaphis B-561* was grown in the presence of 0.1% (w/v) glucose and 1% waste oil (v/v) as carbon sources (section 2.5) and total protein preparations were prepared as described above (section 2.6). Polymer films were prepared as described in section 2.2 and 2.3 and afterward mixed with agar (final concentration 1%, w/v) in 1:1 ratio, poured into a sterile glass Petri dish and subsequently allowed to solidify. Wells were created in the solid media using the wider bore end of a sterile glass Pasteur pipette. The wells were loaded with 50 µl of total protein preparations. Samples were incubated at 37$^{o}$C for 5 days (Teeraphatpornchai *et al.*, 2003).

**2.9. Enzymatic degradation of PCL and mcl-PHA polymers using total protein preparations**

Experiments of enzyme degradation were performed in duplicates with PCL and mcl-PHA polymer samples and total protein preparations of selected *P. chlororaphis B-561* and *Streptomyces* sp. BV315 in PBS buffer pH 7.4, using the same buffer as a control. PCL and mcl-PHA polymers were incubated for 4 weeks at 37°C with shaking at 180 rpm. Polymer films were sterilized with ethanol (70%, v/v) and air-dried under sterile conditions prior to use. Total protein extracts in 5 mL aliquots were added once per week. At the end of the degradation experiments, polymer samples were gently wiped with cotton wool and ethanol (70%, v/v) and weighed.

### 2.10. Biodegradation of PCL and mcl-PHA polymers in laboratory compost model system

Biodegradation of PCL and mcl-PHA polymer films was carried out in compost model system developed in our laboratory, under a constant ambient temperature of 37°C, with liquid cultures of selected *P. chlororaphis B-561* and *Streptomyces* sp. BV315 and a mixture of these two strains, all grown to the exponential phase in minimal media supplemented with glucose (0.1% w/v) and waste cooking oil (1.0 % w/v) as carbon source (Section 2.5). Changes in the appearance of the polymers before and after the biodegradation test were observed. The quantity of bacterial cells was calculated to achieve $1 \times 10^4$ cells per gram of compost. After the addition of the bacterial culture, compost was thoroughly mixed using a sterile spatula. The experiment was set up in glass Petri dish (14 cm diameter, 2 cm height) and 100 g of compost inoculated with bacterial cultures was placed into a Petri dish. Polymer films were placed inside the compost at a depth of 1 cm. The Petri dish was incubated at 37°C over 4 weeks for PCL and 8 weeks for the mcl-PHA polymer. A fresh aliquot of cultures was added at the beginning of each week (5 mL) in order to ensure a constant level of bacterial activity and moisture. A sterile compost that was autoclaved was used as a control in which 5 mL of 20 mM sodium phosphate buffer pH 7.4 was added to ensure similar moisture content as in Petri dishes with tested polymers.

### 2.11. ATR- infrared spectroscopy (ATR-FTIR)

Degraded PCL and mcl-PHA films were characterized by FTIR using an IR-Affinity spectrophotometer (SHIMADZU, Japan). The number of scans was 40, collected in the range of 4000 to 400 $cm^{-1}$ with a spectral resolution of 4 $cm^{-1}$ at room temperature. Data collected by FTIR-ATR were used to estimate changes in chemical structure and crystallinity during the degradation of polymer samples. Carbonyl index (CI) was calculated from the intensity ratio of the absorbance peak of carbonyl at 1720 $cm^{-1}$ to that of $CH_2$ at 1398 $cm^{-1}$, while the

intensity ratio of absorbance peaks at 1294 and 1167 $cm^{-1}$ were used for calculation of the crystallinity index.

### 2.12. Light microscopy

Changes in surface morphology of degraded PCL and mcl-PHA films were followed using a light microscope (Leica DM ILM) with reflected light, equipped with a CCD digital camera (at 100 x magnification).

## 3. Results and discussion

### 3.1. Selection of strains with lipase (esterase) activity

With the aim of testing the ability of *Pseudomonas* and *Streptomyces* strains to degrade PCL and mcl-PHA when grown on waste oil as a carbon source, 10 *Pseudomonas* and 20 *Streptomyces* strains from a laboratory collection were selected and tested for lipase/esterase activity on solid media supplemented with waste cooking oil as a sole carbon source and Rhodamine B as an indicator of lipase enzyme activity. Two strains, *Pseudomonas chlororaphis* B-561, and *Streptomyces* sp. BV315, showing the largest zones on solid media with Rhodamine B were chosen for further tests (Figure 1 inlet). Degrading activities of both strains grown on glucose and waste cooking oil were tested in cell-free extracts, supernatants and in total protein preparation (adjusted concentrations of total proteins in cell-free extracts and supernatants combined) on solid media supplemented with PCL and mcl-PHA and in phosphate buffer using *p*-nitrophenylpalmitate (p-NPP) as a lipase (esterase) model substrate (Figure S1). Larger degradation zones were visible in solid media supplemented with PCL when strains were grown on waste cooking oil compared to glucose (Figure S1 a). Similarly, higher lipase activities (p-NPP assay) were observed from both strains when grown on waste cooking oil compared to glucose-grown cells (Figure S1 b). This is in concordance with the literature data since it has been shown that cooking oils induce lipase (esterase) enzyme expression (Zhang *et al.*, 2009). Specific lipase (esterase) activity of both strains when grown on waste cooking oil as a sole carbon source, was assayed in time, and *Streptomyces* sp. BV315 exhibited 1.8 times higher specific enzyme activity than that of *P. chlororaphis* B-561 (Figure 1). Since both cell-free extracts and supernatants of *Pseudomonas chlororaphis* B-561 and *Streptomyces* sp. BV315 showed considerable *in-vitro* PCL and mcl-PHA degradation activity, total protein preparation containing both intracellular and extracellular proteins of strains grown on waste cooking oil as carbon source were used in all further experiments.

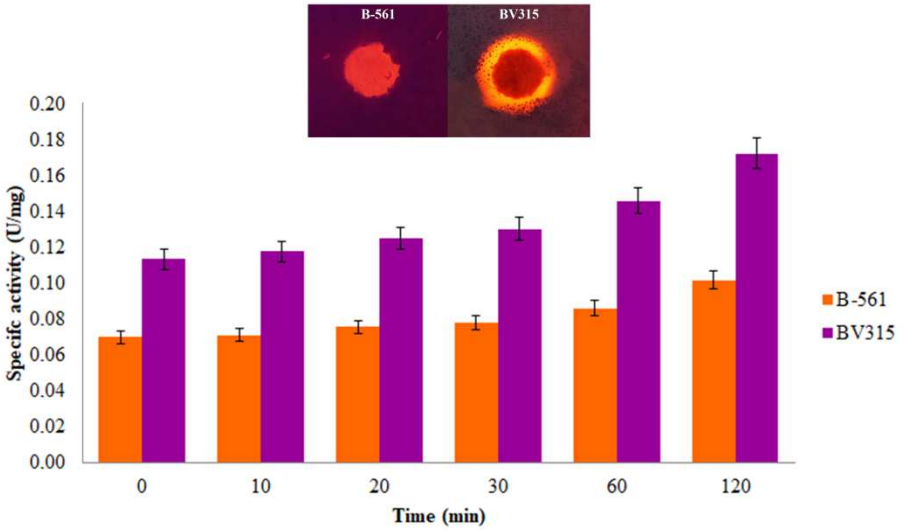



**Figure 1.** Lipase activity of selected *Pseudomonas chlororaphis* B-561 and *Streptomyces* sp. BV315 grown on solid media with waste cooking oil as substrate. Rhodamine B was used as a qualitative indicator of enzyme activity (photo inlet) on agar plates while a quantitative colorimetric assay using p-nitrophenol palmitate (p-NPP) as a substrate was used for liquid grown cultures.

Degradation of oil- and bio-based polymers by microorganisms involves esterases such as lipases and cutinases, and in the case of mcl-PHA, specific PHA depolymerases are employed as well. *Alcaligenes faecalis* and *Pseudomonas aeruginosa* PAO1 have been reported to express lipases with PCL-degrading activity (Oda *et al.*, 1997, Ponjavic *et al.*, 2017a). Cutinases, predominantly of fungal origin have been reported to degrade biopolymers (Dimarogona *et al.*, 2015). In addition, lipase from *Bacillus subtilis* was reported to degrade PHA synthetised by *Enterobacter* sp. (Palanisamy *et al.*, 2016). Most described PHA depolymerases are specific for scl-PHA with an only a limited number of strains reported to express mcl-PHA depolymerases. Several mcl-PHA depolymerases have been described in *Pseudomonas* spp. (Kim et al., 2002, Kim et al., 2007, Anis et al., 2017) and few in *Streptomyces* spp. (Chua *et al.*, 2013, Santos *et al.*, 2013, Martinez *et al.*, 2015). We have resorted to *Pseudomonas* and *Streptomyces* spp. from the laboratory collection of non-

pathogenic soil isolates, as representatives of these two bacterial genera were characterized as the best producers of enzymes for biopolymer degradation including lipases and PHA-depolymerases (Jaeger & Rosenau, 2004, Spasic *et al.*, 2018), therefore showing the promise as tools for biopolymer waste treatment.

### 3.2. Polymer degradation using total protein preparations of *P. chlororaphis* B-561 and *Streptomyces* sp. BV315

PCL and mcl-PHA films approximately the same surface area (10 × 20 mm, 40 mg for PCL and 200 mg for mcl-PHA) were used for biodegradation experiments in PBS (pH 7.4) with adjustments so that protein concentration in total protein preparations (cell-free extracts and culture supernatants) from *P. chlororaphis* B-561 and *Streptomyces* sp. BV315 were approximately the same (Table 1). Fresh total protein preparations were added every week. After 2 and 4 weeks polymer films were cleaned, air-dried and weighed (Table 1). Upon 4-week exposure to total protein extracts from tested strains, both materials showed surface erosion with a moderate weight loss of 1 - 1.3% when *P. chlororaphis* B-561 was utilized on both materials (Table 1). Ponjavic et al. used cell free extracts, containing intracellular fraction of the proteins, from the opportunistic pathogen *Pseudomonas aeruginosa* PAO1 and obtained similar weight loss of high molecular weight PCL polymer (Ponjavic *et al.*, 2017b, Ponjavic *et al.*, 2017a). Hermanova and co-workers reported degradation of PCL in phosphate buffer by commercial lipase from fungus *Aspergillus orizae*, with 3.3% of weight loss after 4 weeks of incubation (Hermanova *et al.*, 2012), which is around 2.2 times higher than the weight loss achieved with total proteins from *P. chlororaphis* B-561. In contrast to our work, the majority of studies dealing with mcl-PHA degradation were performed using liquid bacterial cultures. Woolnough and co-workers obtained 40% of polyhydroxybutirate (PHB) film weight loss after 8 days of incubation with liquid bacterial culture (Woolnough *et al.*, 2008).

Although the specific lipase (esterase) activity on model substrate p-NPP was higher for the strain *Streptomyces* sp. BV315 compared to *P. chlororaphis* B-561 (Figure 1), no significant change in weight of PCL nor mcl-PHA was observed using total protein preparations from this strain in degradation experiments in PBS buffer (Table 1).

**Table 1** PCL and mcl-PHA polymer films weight change in degradation experiments with total protein extracts in buffer over 4 weeks.

| Sample | Weight loss, % [a] | Carbonyl index (CI) [b] | Crystallinity index, % [b] |
|---|---|---|---|
| *Liquid assay 4 weeks* | | | |
| PCL_B-561 | 1.02 | 3.5 | 60 |
| PCL_BV315 | 0.76 | 3.4 | 62 |
| PCL_control | 0.0 | 3.2 | 62 |
| PHA_B-561 | 1.35 | | |
| PHA_BV315 | +0.8 | | |
| PHA_control | 0.0 | | |

[a] Values are mean of two experiments carried out in duplicate (standard errors of the weighed masses ranged from 1% to 3%)
[b] untreated PCL had CI=3.5 and crystallinity index 61%

While *P. chlororaphis* B-561 caused weight loss of mcl-PHA films, treatment with *Streptomyces* sp. BV315 led to a weight gain (Table 1), yet light microscopy revealed erosion of polymer surface indicating that this strain also degrades mcl-PHA (Figure 2). Weight gain could be explained by biofilm formation or some other form of deposited material on the polymer surface, which was clearly visible (Figure 2 b). Even though total protein preparations and not bacterial cultures were used in this experimental setup, it might be that a spore from *Streptomyces* sp. BV315 remained intact after protein preparation, causing growth and biofilm formation on the polymer surface. Biofouling phenomenon in PHA degradation was previously studied by Woolnough and co-workers who observed that increase in the biofilm formation causes greater polymer degradation (Woolnough *et al.*, 2008). Changes in surface morphology and erosion of the polymer surface could be detected in films of both materials (Figure 2). From the recorded micrographs it can be seen that PCL before exposure to bacterial protein preparations forms spherulites with clearly distinguished boundaries

between them. PCL exposed to bacterial protein preparations showed surface erosion resulting in different morphology with less visible boundaries between the spherulites (Figure 2 a). Mcl-PHA polymer films also underwent surface degradation and changes in surface morphology compared to non-treated sample. In addition, thin cracks were also noticed on the surface of mcl-PHA films (Figure 2 b).

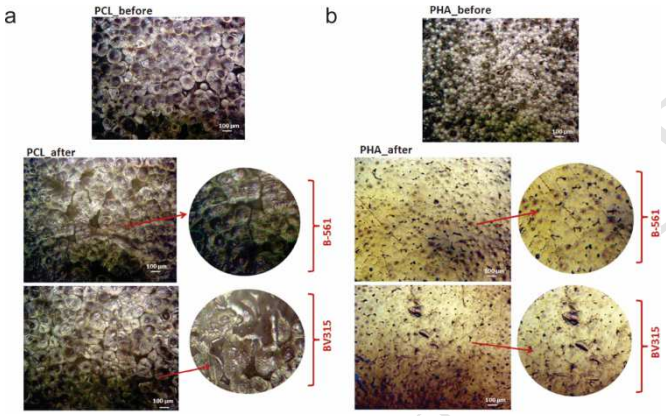



**Figure 2.** PCL (a) and mcl-PHA (b) films before and after 4-week degradation experiments using total protein extracts of *P. chlororaphis* B-561 and *Streptomyces* sp. BV315 in phosphate buffer pH 7.4, assessed by light microscopy.

Changes in the crystalline structure of the surface of PCL films was observed by FTIR analysis following the changes in carbonyl and crystallinity indexes, calculated from the intensity ratio of characteristic peaks that correspond to amorphous and crystalline regions of PCL (Figure S2, Table 1). Based on the small change of up to 3% in CI and up to 1% change for crystallinity index in comparison to the untreated PCL (CI=3.5 and crystallinity index 61%) it could be concluded that during 4 weeks of treatment no substantial changes in the chemical structure and morphology of the surface (amorphous vs. crystalline) had occurred. This is consistent with small weight losses observed during this period. Due to the amorphous structure of mcl-PHA polymers, it was not possible to calculate their CI and crystallinity index.

### 3.3. Polymer degradation in laboratory compost model system using *P. chlororaphis* B-561 and *Streptomyces* sp. BV315 cultures

Polymer degradation was further evaluated in a compost model system using cultures of *P. chlororaphis* B-561 and *Streptomyces* sp. BV315 grown on waste cooking oil as a sole carbon source. PCL and mcl-PHA films (10 × 20 mm, 40 mg for PCL and 200 mg for mcl-PHA) were buried in compost (pH 7.5) and inoculated with a liquid culture of *P. chlororaphis* B-561, *Streptomyces* sp. BV315, and a mixture of both strains adjusted to achieve $10^4$ cells per 1 g of compost. Fresh bacterial cultures were added to compost every week for a total of four weeks for PCL and 8 weeks for mcl-PHA polymer films. During composting experiment temperature in compost was also monitored. At the end of biodegradation experiment, changes in polymer weight were measured while surface degradation was recorded by an optical microscope.

Bacterial activities in compost had effect only on polymer degradation, and did not significantly raise temperature in compost. Compost temperature ranged from 39 °C to 43 °C, which is below both polymer melting temperatures. Both tested polymers showed significant weight loss in compost model system, ranging from 5.7 to 17.7% for the compost model augmented with selected bacterial strains (Table 2). Overall, PCL was more readily degradable in comparison to mcl-PHA. Incubation of strain B-561 with PCL in the compost model resulted in 15.5% weight loss within 4 weeks (Table 2). Interestingly, significant weight loss of 11.7% was detected in compost with indigenous microorganisms, while no weight loss occurred in the sterile compost under tested conditions (Table 2). Although competition with indigenous microorganisms was not observed when single strains were used, the mixture of tested strains caused 2 times lower weight loss of PCL polymer in comparison to single strains used, indicating that tested microorganisms could be competing for nutrients. Rutkowska and co-workers used compost augmented with activated sludge for PCL degradation (Rutkowska *et al.*, 2002) and obtained 2.8 times greater weight loss in

comparison to *Streptomyces* sp. BV315. Narancic and co-workers showed complete degradation of PCL in soil augmented with mature compost after 20 weeks of exposure (Narancic *et al*., 2018). Funabashi and collaborators reported significantly higher percentage of degraded PCL (60% degradation after 3 weeks) but the polymer was crushed into powder and the degradation was carried out in controlled compost at 58°C which is only a few degrees lower than the melting temperature of PCL (Funabashi *et al*., 2009).

Weight loss of mcl-PHA polymer films was observed both in compost model with indigenous microorganisms as well as in sterile compost (Table 2), but it was clear that biodegradation was accelerated with microorganisms since almost 2 times greater weight loss was observed when composting with tested strains. Mcl-PHA degradation in laboratory compost model system was slightly better when *Streptomyces* sp. BV315 was used, causing 13.1% weight loss in comparison to *P. chlororaphis* B-561 which caused 12.1%. Similarly, as for PCL, a mixture of both tested strains led to a decrease in weight loss of mcl-PHA polymer (1.3 times lower weight loss compared to single strains) thus pointing to an antagonism between the tested strains. Literature data regarding biodegradation and composting of PHA is available for scl-PHAs, short-chain length polymers, predominantly PHB and only a few studies are dealing with degradation of mcl-PHA. Lim and co-workers carried out experiments for 112 days, and assessed degradation potential of indigenous microorganisms for mcl-PHA with a weight loss of 16.7% in acidic forest soil, 3% in alkaline forest soil and 4.5% in mangrove forest soil (Lim *et al.*, 2005). Narancic and co-workers were testing degradation of polyhydroxyoctanoate (PHO) in soil augmented with mature compost and PHO remained almost intact after two years exposure in soil (Narancic *et al*., 2018). Our experiments in compost model lasted for 56 days and 13.1% of mcl-PHA weight loss was achieved when compost was augmented with *Streptomyces* sp. BV315.

Light microscopy of polymer films degraded in compost revealed surface erosion for both PCL and mcl-PHA films (Figure 3a and Figure 4a). Treatment of PCL with *P. chlororaphis* B-561 and *Streptomyces* sp. BV315 caused complete disintegration in surface morphology and the spherulites were barely visible (Figure 3a). When a mixture of tested strains was used, spherulite morphology of PCL films remained relatively preserved, which coincides with the smaller weight loss. In the photographs of PCL films treated with single strains for four weeks, greater degraded domains can be observed in comparison to films treated with a mixture of both tested strains (Figure 3b). In order to estimate changes in the crystalline structure of PCL films after biodegradation, CI and crystallinity indexes were also calculated for samples degraded in compost model system. All peaks in FTIR spectrum inherent to PCL were preserved after degradation (Figure S2), and no substantial changes in CI values for any of the degraded samples in comparison to the untreated PCL (3.5) were detected. Most often observed degradation pathway of highly crystalline polymers is through degradation of less ordered (amorphous) domains first, followed by the degradation of highly ordered (crystalline) ones, which could be detected by the change in crystallinity index during the course of degradation. In this study, these changes were recorded only for the samples degraded in the compost without the addition of tested strains (PCL_control with 11.7% weight loss and PCL_sterile control with no weight loss). All the samples in the compost treated with *P. chlororaphis* B-561 and *Streptomyces* sp. BV315 did not show any substantial changes in the crystallinity index compared to untreated PCL (61%). This observation, together with the relatively high weight losses of these samples, implies that the degradation promoted by tested microorganisms goes through progressive degradation of both crystalline and amorphous regions of the polymer film. Despite the great weight losses of the composted films and obvious disintegration of samples and surface erosion (micrographs and photos), crystallinity indexes remained unchanged. This can be attributed to the non-homogeneous

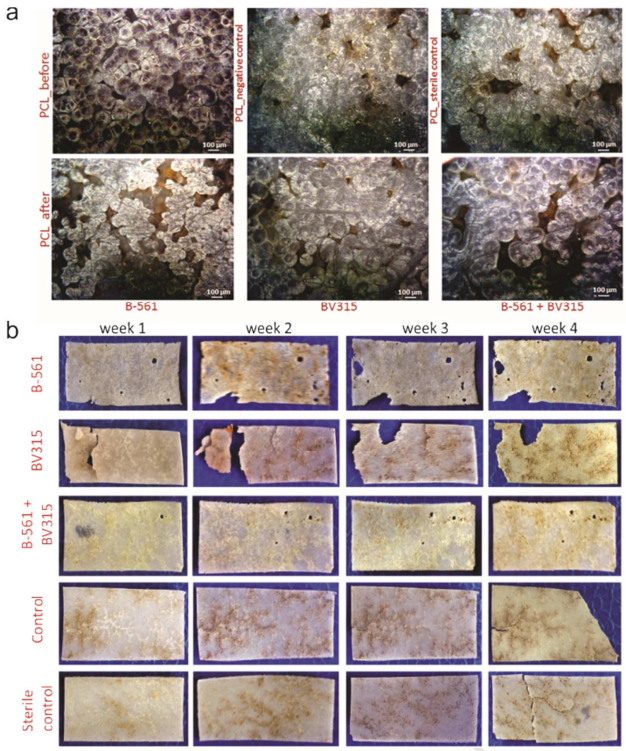
degradation pathway, hence some parts of the films were completely ruined (holes) and on the other, which stayed intact ATR-FTIR analysis was done. PCL films in compost without the addition of tested strains (sterile control and control with indigenous microorganisms) did not exhibit such progressive disintegration and significant decrease in crystallinity indexes (from 61 to 54%) that could be taken as a proof that amorphous regions of PCL polymer films were preferentially degraded while the crystalline regions remained unchanged after four weeks of degradation.

**Table 2** PCL and mcl-PHA polymer films weight change in degradation experiments in laboratory compost model system.

| **Sample** | **Weight loss, %** | **CI** [b] | **Crystallinity index, %** [b] |
|---|---|---|---|
| *Compost 4 weeks* | | | |
| PCL_B-561 | 15.3[a] | 3.3 | 61 |
| PCL_BV315 | 27.7 | 3.5 | 60 |
| PCL_B-561+BV315 | 8.5 | 3.5 | 60 |
| PCL_control | 11.7 | 3.5 | 56 |
| PCL_sterile control | 0 | 3.5 | 54 |
| *Compost 8 weeks* | | | |
| PHA_B-561 | 12.1 | | |
| PHA_BV315 | 13.1 | | |
| PHA_B-561+BV315 | 9.8 | | |
| PHA_control | 5.7 | | |
| PHA_sterile control | 6.2 | | |

[a] Values are mean of two experiments carried out in duplicate (standard errors of the weighted masses ranged from 1% to 3%)

[b] Untreated PCL had CI=3.5 and crystallinity index 61%




476

477 **Figure 3.** Degradation of PCL polymer films in the laboratory compost model system for 4
478 weeks using compost augmented with *P. chlororaphis* B-561 and *Streptomyces* sp. BV315, as
479 well as a mixture of these two strains. Light microscopy images (a) and photos (b).

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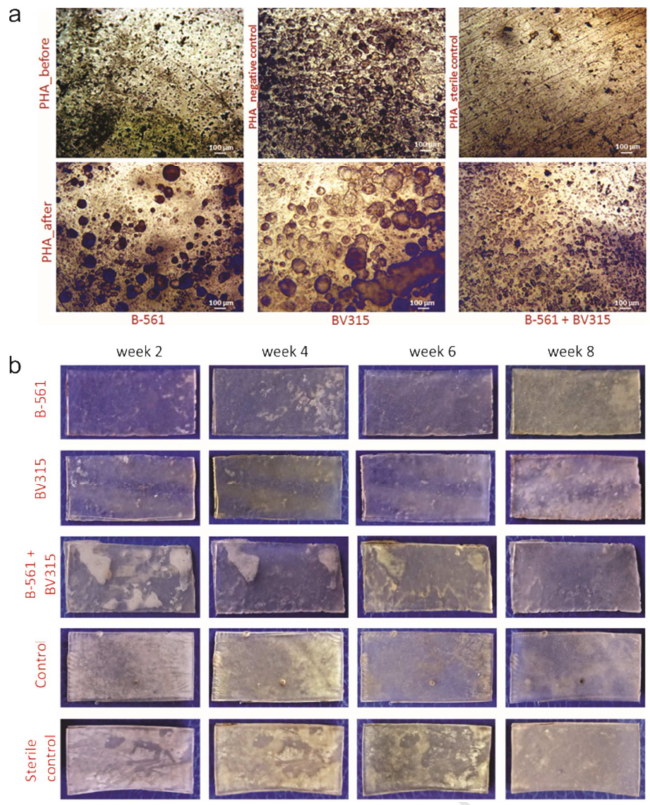


**Figure 4.** Degradation of mcl-PHA films in laboratory compost model system for 8 weeks using compost augmented with *P. chlororaphis* B-561 and *Streptomyces* sp. BV315, as well as a mixture of these two strains. Light microscopy images (a) and photos (b).

Micrographs of mcl-PHA films from compost also showed changed morphology with cracks on polymer surface when single strains were used, while in the case of degradation with a mixture of *P. chlororaphis* B-561 and *Streptomyces* sp. BV315 surface morphology of mcl-PHA films was almost unchanged (Figure 4a). Although light microscopy and weight loss showed degradation of mcl-PHA films in a model compost system, photographs of mcl-PHA films after eight weeks in compost showed only slight changes in the appearance of polymer (Figure 4a). It has been shown that a number of parameters can influence the rate of biodegradation of various biopolymers, including PHA. These include the type of environment, microbial population, the availability of water, temperature, the shape and thickness of the material made of PHA, surface texture, porosity and crystallinity, and the

presence of other components in the material, such as fillers or coloring agents (Lee & Choi, 1999). Prior to our study, Matavulij and Molitoris conducted an interesting study using scl-PHA based material BIOPOL® (Zeneca BioProducts) biodegradation at 11 different sites over a period of 50 weeks (Matavulj & Molitoris, 2000). The highest biodegradation rates appeared in compost (100 % after 10 weeks), greenhouse peat (100 % after 40 weeks), municipal landfill (100 % after 45 weeks) and in a sewage plant under aerobic (100 % after 50 weeks) and anaerobic (98 % after 50 weeks) conditions (Matavulj & Molitoris, 2000).

## 4. Conclusions

Although, there is still no universal definition of material biodegradability under composting conditions, current ASTM D6400 guidelines state that it should completely break down and return to nature as $CO_2$, water, inorganic compounds and biomass, and that at least 90% of material should be degraded within 6 months of composting (ASTM, 1999). PCL and mcl-PHA, considered biodegradable polymers, showed not to be readily degradable by microorganisms and their enzymes under described conditions that resemble small-scale home composting facilities. In this study, moderate weight loss was achieved for both polymers and also mcl-PHA showed two times slower degradation rate than PCL. However, it was shown that *P. chlororaphis* B-561and *Streptomyces* sp. BV315, grown on waste cooking oil could be used for compost bioaugmentation, in order to enhance PCL and mcl-PHA biodegradation and composting process which can further have implications on the more successful management of municipal waste.

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**Figure S1.** Lipase/esterase activity of *P. chlororaphis* B-561 and *Streptomyces* sp. BV315 and when grown on cooking oil or glucose as a carbon source on solid media with PCL in the assay with p-NPP (CFE – cell-free extract; SUP – culture supernatant and TPP – total protein preparation adjusted concentration of total proteins from CFE and SUP).

**Figure S2.** FTIR analysis of degraded PCL polymer films: a) in phosphate buffer using total protein extract and b) in model compost.

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660

**Highlights**

- *Pseudomonas* and *Streptomyces* grown on waste cooking oil for biopolymers degradation
- Enzymes from *Pseudomonas chlororaphis* B-561 hydrolyzed PCL better than mcl-PHA
- Mcl-PHA degraded more efficiently in model compost with *Streptomyces* sp. BV315