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Biodegradation of poly( $\epsilon$ -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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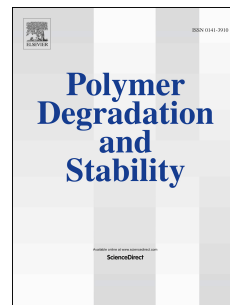
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1 **Biodegradation of poly( $\epsilon$ -caprolactone) (PCL) and medium chain length**  
2 **polyhydroxyalkanoate (mcl-PHA) using whole cells and cell free protein preparations of**  
3 ***Pseudomonas* and *Streptomyces* strains grown on waste cooking oil**

4  
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33 Running title: PCL and mcl-PHA degradation with bacteria grown on waste cooking oil

- 34 Abbreviation list
- 35 **ATR-FTIR** *Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy*
- 36 **PHA** *polyhydroxyalkanoates*
- 37 **scl-PHA** *short-chain length polyhydroxyalkanoates*
- 38 **mcl-PHA** *medium-chain length polyhydroxyalkanoates*
- 39 **lcl-PHA** *long-chain length polyhydroxyalkanoates*
- 40 **PHB** *polyhydroxybutyrate*
- 41 **PCL** *poly( $\epsilon$ -caprolactone)*

42 **Graphical abstract**43  
44

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45 **Abstract**

46 Petrochemical plastics are generally recalcitrant to microbial degradation and accumulate in  
47 the environment. Biodegradable polymers obtained synthetically like poly( $\epsilon$ -caprolactone)  
48 (PCL) or polyhydroxyalkanoates (PHA), obtained biotechnologically, have shown great  
49 potential as a replacement for petroleum-based plastics. Nevertheless, their biodegradation  
50 and environmental faith have been less examined. In this study, thin films of PCL  
51 (200  $\mu\text{m}$ ) and medium chain length PHA (mcl-PHA, 70 molar fraction of  
52 3-hydroxyoctanoate and 30 molar fraction of 3-hydroxydecanoate, 600  $\mu\text{m}$ ) were exposed to  
53 total protein preparations (extracellular proteins combined with a crude cell extract) of soil  
54 isolates *Pseudomonas chlororaphis* B-561 and *Streptomyces* sp. BV315 that had been grown  
55 on waste cooking oil as a sole carbon source. Biodegradation potential of two polyesters was  
56 evaluated in buffer with total protein preparations and in a laboratory compost model system  
57 augmented with selected bacteria. Overall, PCL showed better biodegradation properties in  
58 comparison to mcl-PHA. Both materials showed surface erosion after 4-weeks of exposure to  
59 total protein preparations of both strains, with a moderate weight loss of 1.3% when *P.*  
60 *chlororaphis* B-561 was utilized. In laboratory compost model system PCL and mcl-PHA  
61 showed significant weight loss ranging from 13 to 17% when *Streptomyces* sp. BV315 culture  
62 was used. Similar weight loss of PCL and mcl-PHA was achieved for 4 and 8 weeks,  
63 respectively indicating slower degradation of mcl-PHA. Growth on waste cooking oil as a  
64 sole carbon source increased the potential of both tested strains to degrade PCL and mcl-  
65 PHA, making them good candidates for augmentation of compost cultures in waste  
66 management of both waste cooking oils and biodegradable polymers.

67

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

69

## 70 1. Introduction

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

94 PCL, a biodegradable polyester, synthesized from a petroleum-based monomer, has a  
95 low melting ( $60^{\circ}\text{C}$  to  $65^{\circ}\text{C}$ ) and glass transition temperature ( $-65^{\circ}\text{C}$  to  $-60^{\circ}\text{C}$ ), good water,  
96 and oil resistance, is easily soluble most often used solvents and is easily processed from the  
97 melt (Woodruff, 2010). It has been shown that it can be degraded by the action of widely  
98 distributed aerobic and anaerobic microorganisms in various ecosystems including soil  
99 (Tokiwa *et al.*, 2009). On the other hand, PHAs are entirely a product of microbial  
100 metabolism, produced in environments with limited essential nutrients and usually  
101 intracellularly accumulated as carbon and energy storage in the form of granules (Prieto,  
102 2016, Narancic & O'Connor, 2017). At present, PHAs are classified in three major classes:  
103 short-chain-length PHAs (scl-PHAs) containing C3-C5 carbon atoms in monomers, medium-  
104 chain-length PHAs (mcl-PHAs) with C6-C14 monomers and long-chain length PHAs (lcl-  
105 PHA) containing monomers with more than 14 carbon atoms which reflects in their material  
106 properties such as crystallinity and thermal properties. As biocompatible and biodegradable  
107 polyesters composed of hydroxylated alkanolic acids, that can be directly produced by a wide  
108 range of microbes from renewable resources, PHAs have emerged as a potential alternative to  
109 synthetic polymers as they are preferable from the perspective of human health and  
110 environment safety (Gross & Kalra, 2002, Alvarez-Chavez *et al.*, 2012, Albuquerque &  
111 Malafaia, 2018).

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

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

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



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

## 146 **2. Materials and methods**

### 147 **2.1. Reagents**

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

### 153 **2.2. Preparation of PCL polymer films**

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

160 The PCL films were prepared from a 6% polymer solution in chloroform (1.5 g of the  
161 polymer sample was dissolved in 25 mL of chloroform) by the solvent casting method in  
162 glass Petri dishes (diameter 10 cm) by leaving the solvent to evaporate 12 h at room  
163 temperature. After solvent evaporation, polymer films were dried in a vacuum oven for 24 h  
164 at room temperature and cut into the rectangles (10 × 20 mm, thickness 200 μm, weight about  
165 40 mg) for the degradation experiments.

### 166 **2.3. Preparation of mcl-PHA polymer films**

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

169 PHA produced by *Pseudomonas putida* KT2440 from fed-batch fermentation by the method  
170 of Davis *et al.* (Davis *et al.*, 2015) using glucose and octanoate as carbon source with Mw of  
171 132,000. The mcl-PHA films were prepared in the glass Petri dishes (diameter 10 cm) by  
172 solvent casting method from a 20% polymer solution in acetone (15 g of the mcl-PHA  
173 polymer obtained from Bioplastech Ltd (Dublin, Ireland) was dissolved in 75 mL of acetone)  
174 leaving the solvent to evaporate for 7 days at room temperature. For the degradation  
175 experiments obtained films were cut into rectangles (10 × 20 mm, thickness 600 µm, the  
176 average mass of 200 mg).

#### 177 **2.4. Media for the growth of *Pseudomonas* and *Streptomyces* species**

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

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

#### 186 **2.5. The growth of bacterial cultures**

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

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

## 196 **2.6. Preparation of total proteins for polymer degradation experiments**

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

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

## 212 **2.7. Lipase (esterase) activity**

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

219 quantitative enzyme assay selected strains were grown both on 0.1% (w/v) glucose and 1%  
220 waste oil (v/v) as carbon sources and assay was performed according to Pinsirodom and  
221 Parkin (Pinsirodom & Parkin, 2001) in 3 mL reaction volume. Reactions were incubated at  
222 37°C with shaking at 180 rpm and absorbance was measured after 10, 20, 30, 60 and 120 min  
223 at 410 nm.

## 224 **2.8. Polymer degradation on agar plates**

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

## 234 **2.9. Enzymatic degradation of PCL and mcl-PHA polymers using total protein** 235 **preparations**

236 Experiments of enzyme degradation were performed in duplicates with PCL and mcl-  
237 PHA polymer samples and total protein preparations of selected *P. chlororaphis B-561* and  
238 *Streptomyces* sp. BV315 in PBS buffer pH 7.4, using the same buffer as a control. PCL and  
239 mcl-PHA polymers were incubated for 4 weeks at 37°C with shaking at 180 rpm. Polymer  
240 films were sterilized with ethanol (70%, v/v) and air-dried under sterile conditions prior to  
241 use. Total protein extracts in 5 mL aliquots were added once per week. At the end of the  
242 degradation experiments, polymer samples were gently wiped with cotton wool and ethanol  
243 (70%, v/v) and weighed.

## 244 **2.10. Biodegradation of PCL and mcl-PHA polymers in laboratory compost model** 245 **system**

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

## 262 **2.11. ATR- infrared spectroscopy (ATR-FTIR)**

263 Degraded PCL and mcl-PHA films were characterized by FTIR using an IR-Affinity  
264 spectrophotometer (SHIMADZU, Japan). The number of scans was 40, collected in the range  
265 of 4000 to 400  $\text{cm}^{-1}$  with a spectral resolution of 4  $\text{cm}^{-1}$  at room temperature. Data collected  
266 by FTIR-ATR were used to estimate changes in chemical structure and crystallinity during  
267 the degradation of polymer samples. Carbonyl index (CI) was calculated from the intensity  
268 ratio of the absorbance peak of carbonyl at 1720  $\text{cm}^{-1}$  to that of  $\text{CH}_2$  at 1398  $\text{cm}^{-1}$ , while the

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

### 271 **2.12. Light microscopy**

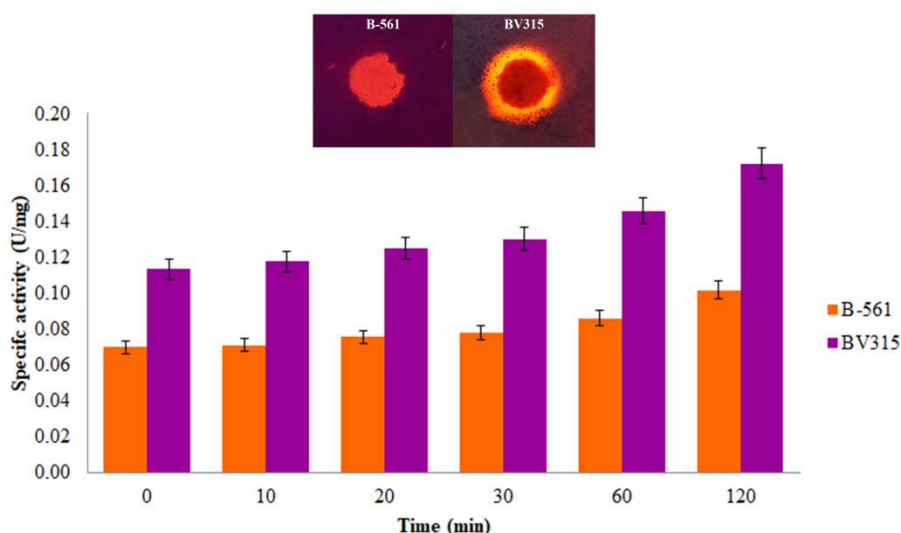
272 Changes in surface morphology of degraded PCL and mcl-PHA films were followed  
273 using a light microscope (Leica DM ILM) with reflected light, equipped with a CCD digital  
274 camera (at 100 x magnification).

275

### 276 3. Results and discussion

#### 277 3.1. Selection of strains with lipase (esterase) activity

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



301  
 302 **Figure 1.** Lipase activity of selected *Pseudomonas chlororaphis* B-561 and *Streptomyces* sp.  
 303 BV315 grown on solid media with waste cooking oil as substrate. Rhodamine B was used as a  
 304 qualitative indicator of enzyme activity (photo inlet) on agar plates while a quantitative  
 305 colorimetric assay using p-nitrophenol palmitate (p-NPP) as a substrate was used for liquid  
 306 grown cultures.

307  
 308 Degradation of oil- and bio-based polymers by microorganisms involves esterases  
 309 such as lipases and cutinases, and in the case of mcl-PHA, specific PHA depolymerases are  
 310 employed as well. *Alcaligenes faecalis* and *Pseudomonas aeruginosa* PAO1 have been  
 311 reported to express lipases with PCL-degrading activity (Oda *et al.*, 1997, Ponjavic *et al.*,  
 312 2017a). Cutinases, predominantly of fungal origin have been reported to degrade biopolymers  
 313 (Dimarogona *et al.*, 2015). In addition, lipase from *Bacillus subtilis* was reported to degrade  
 314 PHA synthesised by *Enterobacter* sp. (Palanisamy *et al.*, 2016). Most described PHA  
 315 depolymerases are specific for scl-PHA with an only a limited number of strains reported to  
 316 express mcl-PHA depolymerases. Several mcl-PHA depolymerases have been described in  
 317 *Pseudomonas* spp. (Kim *et al.*, 2002, Kim *et al.*, 2007, Anis *et al.*, 2017) and few in  
 318 *Streptomyces* spp. (Chua *et al.*, 2013, Santos *et al.*, 2013, Martinez *et al.*, 2015). We have  
 319 resorted to *Pseudomonas* and *Streptomyces* spp. from the laboratory collection of non-



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

### 324 **3.2. Polymer degradation using total protein preparations of *P. chlororaphis* B-561 and** 325 ***Streptomyces* sp. BV315**

326 PCL and mcl-PHA films approximately the same surface area (10 × 20 mm, 40 mg  
327 for PCL and 200 mg for mcl-PHA) were used for biodegradation experiments in PBS (pH  
328 7.4) with adjustments so that protein concentration in total protein preparations (cell-free  
329 extracts and culture supernatants) from *P. chlororaphis* B-561 and *Streptomyces* sp. BV315  
330 were approximately the same (Table 1). Fresh total protein preparations were added every  
331 week. After 2 and 4 weeks polymer films were cleaned, air-dried and weighed (Table 1).  
332 Upon 4-week exposure to total protein extracts from tested strains, both materials showed  
333 surface erosion with a moderate weight loss of 1 - 1.3% when *P. chlororaphis* B-561 was  
334 utilized on both materials (Table 1). Ponjavic *et al.* used cell free extracts, containing  
335 intracellular fraction of the proteins, from the opportunistic pathogen *Pseudomonas*  
336 *aeruginosa* PAO1 and obtained similar weight loss of high molecular weight PCL polymer  
337 (Ponjavic *et al.*, 2017b, Ponjavic *et al.*, 2017a). Hermanova and co-workers reported  
338 degradation of PCL in phosphate buffer by commercial lipase from fungus *Aspergillus orizae*,  
339 with 3.3% of weight loss after 4 weeks of incubation (Hermanova *et al.*, 2012), which is  
340 around 2.2 times higher than the weight loss achieved with total proteins from *P. chlororaphis*  
341 B-561. In contrast to our work, the majority of studies dealing with mcl-PHA degradation  
342 were performed using liquid bacterial cultures. Woolnough and co-workers obtained 40% of  
343 polyhydroxybutyrate (PHB) film weight loss after 8 days of incubation with liquid bacterial  
344 culture (Woolnough *et al.*, 2008).

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

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

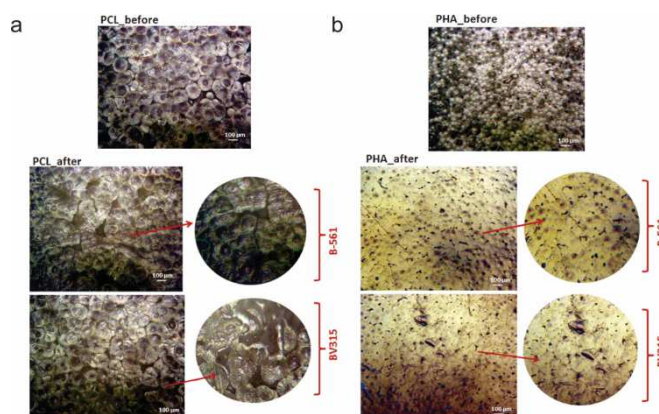
Sample	Weight loss, % <sup>a</sup>	Carbonyl index (CI) <sup>b</sup>	Crystallinity index, % <sup>b</sup>
<i>Liquid assay 4 weeks</i>			
PCL_B-561	1.02	3.5	60
PCL_BV315	0.76	3.4	62
PCL_control	0.0	3.2	62
PHA_B-561	1.35		
PHA_BV315	+0.8		
PHA_control	0.0		

351 <sup>a</sup> Values are mean of two experiments carried out in duplicate (standard errors of the weighed  
 352 masses ranged from 1% to 3%)

353 <sup>b</sup> untreated PCL had CI=3.5 and crystallinity index 61%

354  
 355 While *P. chlororaphis* B-561 caused weight loss of mcl-PHA films, treatment with  
 356 *Streptomyces* sp. BV315 led to a weight gain (Table 1), yet light microscopy revealed erosion  
 357 of polymer surface indicating that this strain also degrades mcl-PHA (Figure 2). Weight gain  
 358 could be explained by biofilm formation or some other form of deposited material on the  
 359 polymer surface, which was clearly visible (Figure 2 b). Even though total protein  
 360 preparations and not bacterial cultures were used in this experimental setup, it might be that a  
 361 spore from *Streptomyces* sp. BV315 remained intact after protein preparation, causing growth  
 362 and biofilm formation on the polymer surface. Biofouling phenomenon in PHA degradation  
 363 was previously studied by Woolnough and co-workers who observed that increase in the  
 364 biofilm formation causes greater polymer degradation (Woolnough *et al.*, 2008). Changes in  
 365 surface morphology and erosion of the polymer surface could be detected in films of both  
 366 materials (Figure 2). From the recorded micrographs it can be seen that PCL before exposure  
 367 to bacterial protein preparations forms spherulites with clearly distinguished boundaries

368 between them. PCL exposed to bacterial protein preparations showed surface erosion  
 369 resulting in different morphology with less visible boundaries between the spherulites (Figure  
 370 2 a). Mcl-PHA polymer films also underwent surface degradation and changes in surface  
 371 morphology compared to non-treated sample. In addition, thin cracks were also noticed on the  
 372 surface of mcl-PHA films (Figure 2 b).



373  
 374 **Figure 2.** PCL (a) and mcl-PHA (b) films before and after 4-week degradation experiments  
 375 using total protein extracts of *P. chlororaphis* B-561 and *Streptomyces* sp. BV315 in  
 376 phosphate buffer pH 7.4, assessed by light microscopy.

377  
 378 Changes in the crystalline structure of the surface of PCL films was observed by FTIR  
 379 analysis following the changes in carbonyl and crystallinity indexes, calculated from the  
 380 intensity ratio of characteristic peaks that correspond to amorphous and crystalline regions of  
 381 PCL (Figure S2, Table 1). Based on the small change of up to 3% in CI and up to 1% change  
 382 for crystallinity index in comparison to the untreated PCL (CI=3.5 and crystallinity index  
 383 61%) it could be concluded that during 4 weeks of treatment no substantial changes in the  
 384 chemical structure and morphology of the surface (amorphous vs. crystalline) had occurred.  
 385 This is consistent with small weight losses observed during this period. Due to the amorphous  
 386 structure of mcl-PHA polymers, it was not possible to calculate their CI and crystallinity  
 387 index.

388 **3.3. Polymer degradation in laboratory compost model system using *P. chlororaphis* B-**  
389 **561 and *Streptomyces* sp. BV315 cultures**

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

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

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

420 Weight loss of mcl-PHA polymer films was observed both in compost model with  
421 indigenous microorganisms as well as in sterile compost (Table 2), but it was clear that  
422 biodegradation was accelerated with microorganisms since almost 2 times greater weight loss  
423 was observed when composting with tested strains. Mcl-PHA degradation in laboratory  
424 compost model system was slightly better when *Streptomyces* sp. BV315 was used, causing  
425 13.1% weight loss in comparison to *P. chlororaphis* B-561 which caused 12.1%. Similarly, as  
426 for PCL, a mixture of both tested strains led to a decrease in weight loss of mcl-PHA polymer  
427 (1.3 times lower weight loss compared to single strains) thus pointing to an antagonism  
428 between the tested strains. Literature data regarding biodegradation and composting of PHA  
429 is available for scl-PHAs, short-chain length polymers, predominantly PHB and only a few  
430 studies are dealing with degradation of mcl-PHA. Lim and co-workers carried out  
431 experiments for 112 days, and assessed degradation potential of indigenous microorganisms  
432 for mcl-PHA with a weight loss of 16.7% in acidic forest soil, 3% in alkaline forest soil and  
433 4.5% in mangrove forest soil (Lim *et al.*, 2005). Narancic and co-workers were testing  
434 degradation of polyhydroxyoctanoate (PHO) in soil augmented with mature compost and  
435 PHO remained almost intact after two years exposure in soil (Narancic *et al.*, 2018). Our  
436 experiments in compost model lasted for 56 days and 13.1% of mcl-PHA weight loss was  
437 achieved when compost was augmented with *Streptomyces* sp. BV315.

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

463 degradation pathway, hence some parts of the films were completely ruined (holes) and on the  
 464 other, which stayed intact ATR-FTIR analysis was done. PCL films in compost without the  
 465 addition of tested strains (sterile control and control with indigenous microorganisms) did not  
 466 exhibit such progressive disintegration and significant decrease in crystallinity indexes (from  
 467 61 to 54%) that could be taken as a proof that amorphous regions of PCL polymer films were  
 468 preferentially degraded while the crystalline regions remained unchanged after four weeks of  
 469 degradation.

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

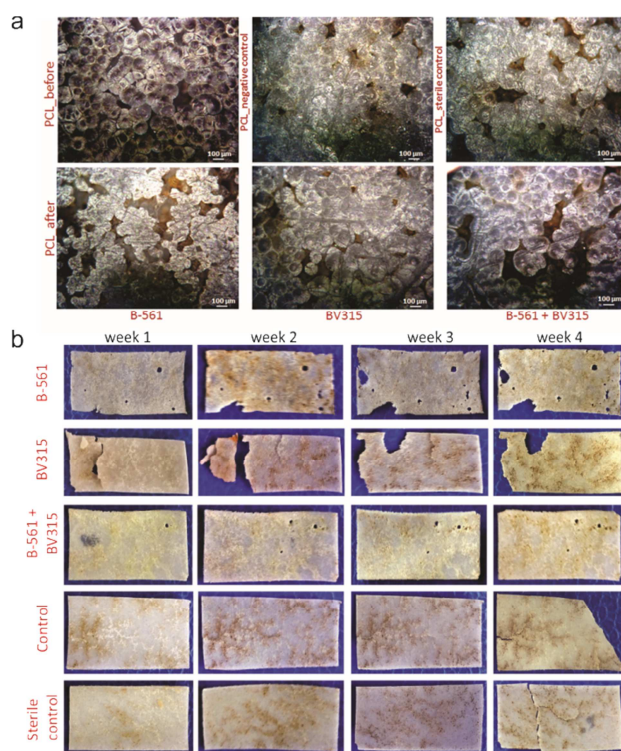
Sample	Weight loss, %	CI <sup>b</sup>	Crystallinity index, % <sup>b</sup>
<i>Compost 4 weeks</i>			
PCL_B-561	15.3 <sup>a</sup>	3.3	61
PCL_BV315	27.7	3.5	60
PCL_B-561+BV315	8.5	3.5	60
PCL_control	11.7	3.5	56
PCL_sterile control	0	3.5	54
<i>Compost 8 weeks</i>			
PHA_B-561	12.1		
PHA_BV315	13.1		
PHA_B-561+BV315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

472 <sup>a</sup> Values are mean of two experiments carried out in duplicate (standard errors of the weighted  
 473 masses ranged from 1% to 3%)

474 <sup>b</sup> Untreated PCL had CI=3.5 and crystallinity index 61%

475





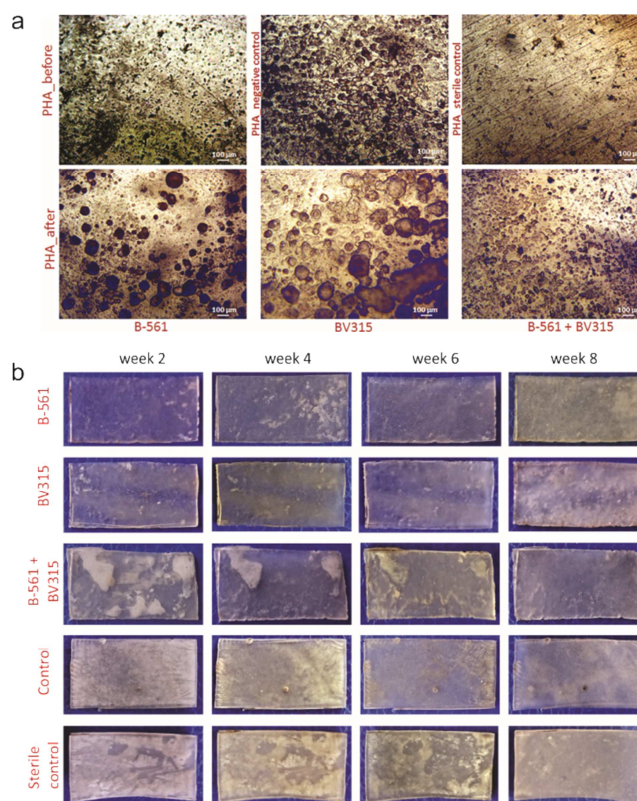
476

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).

480

481





482

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

486

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

497 presence of other components in the material, such as fillers or coloring agents (Lee & Choi,  
498 1999). Prior to our study, Matavulij and Molitoris conducted an interesting study using scl-  
499 PHA based material BIOPOL® (Zeneca BioProducts) biodegradation at 11 different sites  
500 over a period of 50 weeks (Matavulj & Molitoris, 2000). The highest biodegradation rates  
501 appeared in compost (100 % after 10 weeks), greenhouse peat (100 % after 40 weeks),  
502 municipal landfill (100 % after 45 weeks) and in a sewage plant under aerobic (100 % after 50  
503 weeks) and anaerobic (98 % after 50 weeks) conditions (Matavulj & Molitoris, 2000).

504

#### 505 **4. Conclusions**

506 Although, there is still no universal definition of material biodegradability under  
507 composting conditions, current ASTM D6400 guidelines state that it should completely break  
508 down and return to nature as CO<sub>2</sub>, water, inorganic compounds and biomass, and that at least  
509 90% of material should be degraded within 6 months of composting (ASTM, 1999). PCL and  
510 mcl-PHA, considered biodegradable polymers, showed not to be readily degradable by  
511 microorganisms and their enzymes under described conditions that resemble small-scale  
512 home composting facilities. In this study, moderate weight loss was achieved for both  
513 polymers and also mcl-PHA showed two times slower degradation rate than PCL. However, it  
514 was shown that *P. chlororaphis* B-561 and *Streptomyces* sp. BV315, grown on waste cooking  
515 oil could be used for compost bioaugmentation, in order to enhance PCL and mcl-PHA  
516 biodegradation and composting process which can further have implications on the more  
517 successful management of municipal waste.

518

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522

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

527  
528 **Figure S2.** FTIR analysis of degraded PCL polymer films: a) in phosphate buffer using total  
529 protein extract and b) in model compost.

530

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**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