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1 **Microbial degradation of low-density polyethylene and**

2 **synthesis of polyhydroxyalkanoate polymers**

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16 Running Title: Biodegradation of LDPE and bioconversion to scl-PHA

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26 **Abstract**

27 We have characterized the ability of eight bacterial strains to utilize powdered low-density
28 polyethylene (LDPE) plastic (un-treated and without any additives) as a sole carbon source. Cell mass
29 production on LDPE containing medium after 21 days of incubation varied between 0.083 ± 0.015 g L^{-1}
30 cell dry weight (cdw) for *Micrococcus luteus* IRN20 and 0.39 ± 0.036 g L^{-1} for *Cupriavidus necator*
31 H16. The percent decrease in LDPE mass ranged from 18.9% ± 0.72 for *M. luteus* IRN20 to 33.7% ±
32 1.2 for *C. necator* H16. Linear alkane hydrolysis products from LDPE degradation were detected in the
33 culture media, and the carbon chain lengths of the hydrolysis products detected varied, depending on
34 the species of bacteria. We also determined that *C. necator* H16 produced short chain length
35 polyhydroxyalkanaote (scl-PHA) biopolymers, while *Pseudomonas putida* LS46 and *Acinetobacter*
36 *pitti* IRN19 produced medium chain length (mcl-PHA) biopolymers while growing on PE powder. *C.*
37 *necator* H16 accumulated poly (3-hydrodxybutyrate-co-3-hydroxyvalerate) (PHB/V) polymers to 3.18
38 % ± 0.4 of cdw. The monomer composition of the PHB/V was 94.9% ± 0.61 3-hydrodxybutyrate and
39 5.03% ± 0.56 3-hydroxyvalerate. This is the first report that provides direct evidence for simultaneous
40 bioconversion of LDPE plastic to biodegradable polyhydroxyalkanoate polymers.

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42 **Keywords:** Low-density polyethylene; Biodegradation;Bioassimilation; Bioconversion;
43 Polyhydroxyalkanoates

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44 **Introduction**

45 From 1950 to 2015, total amount of synthetic plastics discarded in landfills or in the natural
46 environment has been estimated at approximately 4,900 megatonnes (Mt). This amounts to about 60%
47 of all the plastic materials ever produced. According to European data, the amount of plastic waste
48 deposited in landfill sites varies from less than 10% of plastic waste generated (in countries where
49 landfill bans are in effect), to nearly 80% in other countries (Geyer et al. 2017; Plastics Europe 2017;
50 Ragaert et al. 2017). Low Density Polyethylene (LDPE) is the most abundant solid plastic waste
51 discarded in landfills, in the form of plastic bags (69.13%). Despite recycling and energy recovery
52 efforts, a lack of proper recycling and reuse facilities resulted in the inundation of both land and the
53 oceans with increasing amounts of plastic wastes, and the harmful impacts of non-biodegradable
54 polyethylene (PE) waste accumulation continue to increase.(Ragaert et al. 2017; Rajasekaran and Maji
55 2018).

56 Over 90 microorganisms, including bacteria and fungi, have been reported to degrade petroleum-
57 plastics, (Jumaah 2017). Most studies of PE biodegradation have reported that bacteria, such as
58 *Rhodococcus* spp. (Bonhomme et al. 2003; Gilan et al. 2004; Fontanella et al. 2010) *Pseudomonas* spp.
59 (Rajandas et al. 2012), *Bacilius* spp. (Abrusci et al. 2013; Sudhakar et al. 2008), and *Cupriavidus*
60 *necator* (also formerly known as *Ralstonia eutropha* H16) (Yoon et al. 2012), and fungi, such as
61 *Aspergillus* and *Fuzarium* (Hassan et al. 2007; Sahebnazar et al. 2010), are able to hydrolyze PE only
62 after UV-, thermal-, or other methods of pre-treatment, which renders the carbon chains of polymer
63 sensitive to biodegradation (Ammala et al. 2011).

64 There is no exact definition for biodegradation because it is a complex process that is dependent on
65 many factors, such as availability of a substrate, morphology, surface characteristics, and molecular
66 weight [Ammala et al. 2011; Albertsson et al. 1987). One key weakness of the term ‘biodegradable’ is
67 that it does not contain any information on the location, time-scale, and extent of the decomposition
68 process. Indeed, biodegradability is often defined in relation to the purpose or the conditions of interest,

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69 with separate standards and test methods (Harrison et al. 2018). Different locations (soil, wastewater,
70 freshwater or marine habitats), and different conditions (*in vitro* or *in vivo*, aerobic or anaerobic), as
71 well as different structures and compositions of the polyethylene substrate, and different inoculae
72 (sources of inocula used for biodegradability tests are diverse and often non-specific) have been applied
73 in biodegradation experiments. Moreover, biodegradation has been quantified by a wide-range of
74 measures, including substrate weight loss, perentage of carbon dioxide emission, and changes in the
75 mechanical properties or the chemical structure of the polyethylene substrate used. This makes
76 comparisons of the results from various biodegradation experiments difficult and indicates that more
77 precise descriptions of the methods and specific measured used are required.

78 Microbial degradation of plastics is caused by certain enzymatic activities that lead to a chain
79 cleavage of the polymer into oligomers and monomers. Biodegradation the PE plastic wastes by
80 microbial isolates and their enzymes has become a major topic of research (Nowak et al. 2011; Kyaw et
81 al. 2012; Gajendiran et al. 2016; Sen and Raut 2016). PE has a simple linear structure of n-alkanes and
82 is extremely resistant to biodegradation. Many of the species shown to degrade polyethylene are also
83 able to hydrolyze and consume linear n-alkanes, like paraffin. Alkane hydroxylases (AHs) are the key
84 enzymes in aerobic degradation of alkanes by bacteria. The linear paraffin molecules (C44H90, Mw
85 618) are known to be consumed by a number of different microorganisms in 20 days [Haines et al,
86 1975]. Microbial hydrolysis of paraffin is well understood, and the initial step involves hydroxylation
87 of C-C bonds to generate primary or secondary alcohols, which are further oxidized to aldehydes or
88 ketones, and then to carboxylic acids, which are hydrophilic (Wanatabe et al. 2003; Alvarez et al.
89 2003).

90 Carboxylated n-alkanes are analogous to fatty acids, which can be catabolized by bacteria via the β-
91 oxidation pathway. These enzymatic oxidation products may be absorbed by microbial cells where
92 they are catabolized (Usha et al. 2011). Aerobic biodegradation of PE degradation by bacteria is
93 thought to occur in four stages; 1) Biodeterioration, whereby oxidative enzymes released by

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94 microorgansims catalyze the formation of carbonyl-groups throughout the linear carbon chain. Further
95 oxidation decreases the number of carbonyl-groups due to the formation of carboxylic acids; 2)
96 Biofragmentation, which results in hydrolysis and fragmentation of the polymer carbon chains, and the
97 release intermediate materials. Surface corrosion of the plastic material occurs in this step; 3)
98 Bioassimilation, where small hydrocarbon fragments are metabolized by the bacterial cells; and 4)
99 mineralization, whereby catbolized hydrolysis products are converted to microbial biomass with the
100 concomitant release of carbon dioxide (CO_2) and water (H_20).

101 Although biodeterioration and biofragmentation have been confirmed and established (Ammala et
102 al. 2011, Albertsson and Karlsson 1990), evidence for bioassimilation and complete mineralization are
103 very limited. In other words, bioassimilation and mineralization of PE have not been confirmed due to
104 a lack of supporting evidence (Sen and Raut 2016; Yang et al. 2014). We report here on the isolation
105 and characterization of several bacteria that are able to utilize LDPE as a sole carbon source, and
106 provide evidence for biofragmentation and bioassimilation of petroleum-derived LDPE into biomass
107 plus biodegradable polyhydroxyalkanoates polymers.

108 Capability of PE biodegradation within some isolates used in this project (*Pseudomonas putida*
109 IRN22, *Acinetobacter pitti* IRN19, *Micrococcus luteus* IRN20) was prevoiusly reported by Montazer et
110 al. (2018). In this work, the percentage weight loss of LDPE and bacterial biomass production were
111 used as indicators of biodegradation and compared among the isolates tested. However, the main object
112 of this work was to provide evidence for microbial fragmentation and conversion (assimilation) of
113 LDPE into polyhydroxyalkanoate polymers by the bacterial cells during biodegradation.

114 **Materials and methods**

115 ***LDPE powder***

116 LDPE powder, with a particle size of 400 µm or less (screen retention of 50 mesh is 4.4%) and a
117 molecular weight range between 20,000 and 150,000, was supplied by Alfa-Asar Company (USA;
118 CAS number 9002-88-4). According to the supplier, the Feedstock Melt Index and density were 3.50

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119 g/10 min (at 190 ^{o}C and 2.16 kg load) and 0.9227 g/cm^3, respectively. According to supplier's data
120 sheet, the polymer was pure and free of stabilizing agents. The PE particles sterilized by exposure to
121 UV-light (254 nm) for 1 hour while mixing.

122 ***Culture media***

123 Bacteria were assessed for their ability to grow on LDPE as a sole carbon source using minimal salt
124 polyethylene (PE-) medium. The composition of the PE medium, per liter (L), was as follows,
125 Na_2HPO_4 5g, KH_2PO_4 2g, $(NH_4)_2SO_4$ 3g, KCl 0.15g, NaCl 0.5g, $CaCl_2$ 1.2 mg, $MgSO_4$ 20mg,
126 Fe(III)NH_4 citrate 1 mg, Trace elements solution 1 mL. Each L of trace element solution contained the
127 following: 0.3 g of H_3BO_3, 0.2 g of $CoCl_2 \cdot 6H_2O$, 0.1 g of $ZnSO_4 \cdot 7H_2O$, 30 mg of $MnCl_2 \cdot 4H_2O$, 30 mg
128 of $NaMoO_4 \cdot 2H_2O$, 20 mg of $NiCl_2 \cdot 6H_2O$, 10 mg of $CuSO_4 \cdot 5H_2O$. Also, 1 mL Tween-80 was added as
129 a biosurfactant to facilitate suspension of the LDPE particles in the media. The addition of surfactants
130 such as Tween-80 and mineral or paraffin oil in the medium has been reported to enhance
131 biodegradation of PE by microorganisms by 50%, due to increased hydrophilicity of PE surface and
132 biofilm formation (Gilan et al. 2004). The other advantage is that Tween-80 is not capable of
133 denaturing proteins/enzymes and can detoxify some unsaturated fatty acids, which would otherwise
134 inhibit microbial growth on the PE surface (Albertsson et al. 1993). The medium was autoclaved first,
135 then the UV-sterilized PE powder was added to the medium in the biosafety cabinet. Ramsay's medium
136 was used with the most active PE degrading bacteria to assess the production of polyhydroxyalkanoates
137 under nitrogen-limited conditions. The final pH for both media was 7.0 (Fu et al. 2015).

138 ***Bacterial strains and culture conditions***

139 Four LDPE-degrading bacteria isolated from plastic-dump landfill soil located in Mashhad, Iran,
140 including *Pseudomonas putida* IRN22 (MF348181), *Acinetobacter pitti* IRN19 (MF348182),
141 *Micrococcus luteus* IRN20 (MF348185), and *Delftia tsuruhatensis* IRN27 (MF348184) were studied in
142 these experiments (Montazer et al. 2018). In addition, we tested four bacteria that are known to
143 synthesize PHA polymers, and for which an annotated genome is available: *Cupriavidus necator* H16

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144 *Pseudomonas putida* LS46, *Pseudomonas chlororaphis* PA23, and *Pseudomonas monteilii* MO_2. These
145 species were available in the laboratory of the corresponding author, Dr. David Levin, at University of
146 Manitoba.

147 These eight bacteria were cultured on paraffin-mimimal salt medium (MSM). Paraffin was used
148 as the initial carbon source in minimal medium cultures to adapt the microbial physiology to LDPE
149 degradation. The structure of LDPE is similar to paraffin, which is known to be biodegradable and can
150 be regarded as the low molecular counter part of synthetic polyolefins (Albertsson and Karlsson 1990).
151 In this step, the concentration of paraffin was decreased gradually in the media and LDPE powder
152 added in increasing amounts in three steps; Step 1) Glucose (0.05%) plus paraffin (1%); Step 2)
153 Paraffin (0.5%) and LDPE (0.5%); and step 3) Paraffin (0.05%) and LDPE (1%). All bacteria were
154 cultured on first medium until turbidity due to cell density was observed. Cells from these cultures (1%
155 v/v) were then transferred to the next media. All media contained 0.1% Tween 80.

156 ***Controls used in these experiments***

157 *Assessment of the effect of UV-irradiation on LDPE structure*

158 The PE powder used as substrate for microbial growth in these experiments was surface sterilized
159 by exposuure to ultraviolet (UV-) light (254 nm) for 1 hour. In order to examine the effect of the UV-
160 irradiation on LDPE structure, and determine the possiblity of that this exposure altered the stucture of
161 the LDPE, rendering it sensitivite to microbial attack, the UV-irradiated LDPE was subjected to FTIR
162 spectroscopy using an Agilent Cary 620 spectrophotometer, equipped with a Zn-Se-Crystal ATR
163 accessory. Analyses of the FTIR sprectra was conducted to evaluate the formation of carbonyl-groups,
164 which would indicate alteration of the LDPE structure. The effect of UV-irradiation on LDPE structure
165 was assessed by measuring the ratio of adsorption at 1701.21 cm^{-1} (which detects carbonyl-group
166 formation) to the 2914.72 cm^{-1} (which detects the CH_2 asymmetric stretch) in three independent
167 replicate experiments, both before and after exposure to 254 nm UV-radiation (Abrusci et al. 2013).

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168 We also compared bacterial growth on un-treated versus UV-irradiated LDPE to determine if the UV-
169 irradiated LDPE was more susceptable to bacterial degradation.

170 *Assessment of the effect of Tween-80 and citrate on microbial growth*

171 Both Tween-80 and citrate in the culture medium could serve as carbon sources for bacterial
172 growth. Therefore, three control cultures were conducted to determine if these media components could
173 support growth: 1) LDPE in the culture medium containing 0.1% Tween-80 and 1 mg/L Fe(III)NH_4
174 citrate without inoculation of bacteria, to test for microbial contamination (i.e. to test the sterility of the
175 irradiated LDPE); 2) LDPE in the culture medium containing 0.1% Tween-80 and 1 mg/L Fe(III)NH_4
176 citrate inoculated with *Escherichia coli* DH5α to determine if carbon sources other than LDPE derived
177 from the LDPE powder may support microbial growth; 3) Culture medium containing 0.1% Tween-80
178 and 1 mg/L Fe(III)NH_4 citrate, with no LDPE, inoculated with each of the bacteria tested in this study,
179 to determine if Tween-80 and Fe(III)NH_4 citrate could support bacterial growth as a sole carbon
180 sources.

181 ***Degradation assays***

182 LDPE powder (100 mg) was added to cylinder tubes containing 10 mL of the sterile basal
183 medium. After inoculation with cultures of the eight species individually, these were shaken on a rotary
184 shaker at 30 ^{o}C. Experiments were performed in triplicate for 3 weeks. Controls consisted of tubes
185 containing medium with no bacterial inoculation. *Escherichia coli* DH5α was also cultured as a control
186 to determine if any additional chemical(s) that could serve as a carbon source were in the PE powder.
187 Bacterial growth was measured over 21 days and PE degradation was monitored by measuring the
188 weight of the PE particles before and after incubation. Cell mass production (cell dry weight) was also
189 measured at the end of the experiment (day 21).

190 ***Cell growth assays***

191 The growth of bacteria on PE particles was measured according to Uchida et al. (2000) as optical
192 density at 600 nm (OD_{600}) with a visible spectrophotometer (model Ultrospec 500 pro; Biochrom,

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193 USA). Before measurement, all tubes were shaken gently to suspend the settled biomass and then
194 allowed to stand for 30 minutes to allow time for the suspended PE particles to float up and out of the
195 path of the optical density reading.

196 ***LDPE weight loss measurement***

197 To accurately determine the dry weight of residual low LDPE (after the 21 days of culturing), PE
198 with bound cells were filtered using a single-fold tissue paper (Tork, SCA Tissue North American
199 LLC, USA). The filter pores were small enough to capture the PE particles, and yet wide enough for
200 the mineral components and cells to be washed through. The filterd PE particles were then washed with
201 2% (w/v) sodium dodecyl sulfate (SDS) to lyse any remaining cells that were adhered to the PE particle
202 surface, and the cell debris was captured, dried, and weighed. The PE particles (on the filter paper)
203 were further rinsed with distilled water, and then dried overnight at 60 ^{o}C before weighing. Although
204 some dried cells debris remained attached to the PE particles (visualized by SEM), their contributon to
205 the total PE particle mass was considered negiligible. The percentage of PE particle weight loss was
206 determined using the formula (kyaw et al. 2012):

207 $$\%\text{weight loss} = ((\text{Initial weight} - \text{Final weight})/\text{Initial weight}) \times 100$$

208 ***Biomass production measurements***

209 The residual media containing microbial biomass were centrifuged at 8000 rpm (7155 x g) in pre-
210 weighed 50 mL Falcon tubes for 30 minutes (model IEC Multi, Electron Corporation Company, USA).
211 The supernatants were collected, and the cell pellets were dried at 60 ^{o}C over night. The tubes
212 containing the dried cell mass were then weighed using a four digit balance (model SL-114, Denver
213 instrument, USA).

214 ***Analysis of LDPE hydrolysis products***

215 The presence of putative hydrolysis products (linear alkanes) in the culture supernatant generated
216 by low density LDPE biodegradation were analyzed by gas chromatography (GC-FID). At the end of
217 the experiment (day 21), triplicate tubes containing the 10 mL bacterial cultures were extracted with 5

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218 mL hexane by shaking for 1 min. After the separation of the layers, the organic layer was analyzed on
219 an Varian CP 3800 gas chromatograph instrument (Varian, Inc., CA) equipped with a FID detector and
220 fitted with a 50 m × 0.2 mm × 0.33 μm CP Sil-CB capillary column (Agilent, Canada) using a split
221 mode (split ratio 5:1). The carrier gas was Helium with volume injection of 5 ml/min and Temprature
222 of 200°C.The oven method employed was 35 °C for 2 min, ramping at 10 °C /min to 250 °C, followed
223 by a ramp of 20 °C /min to 320 °C and held at this temperature for 23 min. For peak identification, a
224 solution containing C7-C40 alkane standards was used (Guzik et al. 2014). In order to calculate the
225 alkane concentrations, standard curves were using C22 to C31 alkanes with concentrations of
226 1:10,1:20, and 1:40 were used to construct the standard curve.

227 ***Scanning Electron Microscopy***

228 In order to survey the biofilm formation, the polyethylene particles were removed from the culture
229 medium after 3 weeks incubation to observe the bacterial colonization of the polyethylene particles and
230 the extent of surface erosion. Samples were prepared according to Harshvardhan and Jha (2013). The
231 samples were washed for 2 min in 0.01 M phosphate buffer (pH 7.2) to release excess medium. In
232 contrast, in the procedure for the examination of surface erosion, polyethylene samples were washed
233 with a 2% SDS solution in water followed by several rinses in warm distilled water to completely
234 remove surface-adhered cells. Both types of polyethylene samples were fixed in 2% glutaraldehyde in
235 phosphate buffer (pH; 7.2) for 2 h, dehydrated in graded ethanol (50%, 70%, and 100%). After fixation,
236 the samples were dried in a vacuum. The dehydrated samples were sputter-coated with gold at 50
237 militorr, 45 miliamps, for 45 seconds which resulted in a 100 angstrom thick gold layer (Denton
238 vacuum Inc, model: desk II, USA). The samples were then examined using a FEI Quanta FEG 650
239 Environmental SEM.

240 ***PHA production by selected LDPE degrading bacteria***

241 *Cupriavidus necator* H16, *Pseudomonas putida LS46,* and *Acinetobacter pitti* IRN19 (all know to
242 be PHAs producing bacteria) were grown in 1L Erlenmeyer flasks containing 500 mL of nitrogen-

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243 limited (1 g/L ammonium sulphate) Ramsay's media and LDPE particles at a final concentration of
244 1%. The flasks were incubated at 30 ^{o} C with shaking at 150 rpm. Biomass was collected by
245 centrifugation and dried at 60 ^{o} C over night for further analysis.

246 ***GC-MASS analysis of Biomass***

247 Accumulation of PHA polymers by *Cupriavidus necator* H16, *Pseudomonas putida* LS46, and
248 *Acinetobacter pitti* IRN19 biomass was analyzed by GC. Oven dried cell mass samples were processed
249 by the acid-catalyzed methanolysis procedure as described by Fu et al. (2014). GC analyses were
250 conducted by using the organic phase on an Agilent 7890A gas chromatograph equipped with a split-
251 splitless inlet (operated in split mode, split ratio 10:1), a DB23 capillary column (Agilent, 30 m × 250
252 µm× 0.25 µm), and a flame ionization detector. Method operating parameters and peak quantification
253 were as described by Fu and et al. (2014). For peak identification, Benzoic acid and DL-β-
254 Hydroxylauric acid (n-dodecanoic acid), purity ≥99% for GC analysis- a C12 fatty acid-CH3
255 (CH2)10CH3) were used as internal standards (Fu et al. 2014; Blunt et al. 2017).

256 ***Statistical analyses***

257 Data for LDPE weight loss, cell growth (OD_{600}) and total hydrolysis products concentration for
258 each bacterium were subjected to statistical analyses using the R Core Team (2017) stats package.
259 Mean variables were compared using the Duncan test, and correlations between variables were
260 calculated by the Pearson Square method at probability level of 5% (R-Core Team 2017).

261 **Results**

262 ***FTIR assessment of UV-irradiated LDPE***

263 Effect of UV irradiation on carbonyl-group production was assessed by FTIR spectroscopy (Table 1).
264 The FTIR chromatograms, for both untreated LDPE and for LDPE that had been exposed to UV-
265 radiation for one hour, showed constant ratios, suggesting that carbonyl-group formation was
266 approximately zero (Fig S1). Thus, UV-irradiation for one hour did not have a significant effect on

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267 LDPE structure. In contrast, it is well documented that exposure of LDPE to UV-radiation for 250 hours
268 or more is able to induce significant levels of carbonyl-group prodution, making LDPE polymers
269 sensitive to microbial degradation (Hassan et al. 2007; Yamada-Onodera et al. 2001; Abrusci et al.
270 2011).

271 ***Bacterial growth on LDPE***

272 The growth rate and final cell densties of bacteria cultured with untreated and UV-irradiated LDPE
273 were not different statistically, confirming that one hour of UV-exposure of LDPE did not affect
274 growth rate and cell densities (data not shown). Also, all control cultures as described in Materials and
275 Method were either negative for bacterial growth (no growth detected) or displayed negligible growth,
276 which if present, was subtracted from main data (shown in Table 2).

277 Culture tubes containing 10 mL PE medium were inoculated from tubes containing 0.05% paraffin
278 and 1% LDPE. The volume of the inoculant was 100 µL (1% of the total culture volume), which means
279 that the amount of carry-over was small. Growth of bacteria on PE- medium was rapid with all bacteria
280 reaching their maximum cell density in 2 to 3 days (Figure 1). The experiments were carried-out over
281 21 days becuase we expected microbial degradation of LDPE to be slow. Thus, the rapid initial growth
282 of some of the tested bacteria (*C. necator* H16, *P. putida* LS46, *P. putida* IRN22, *P. choroaphis* PA63,
283 *P. montielllii* MO2, *A. pitti* IRN19) to high cell densities in 2 to 3 days was an unexpected result. Cell
284 densities of two the tested bacteria (*D. tsuruhatensis* IRN27 and *M. luteus* IRN20) peaked at lower
285 levels in the first 2 to 3 days compared with the other bacteria.

286 After day 3, the bacteria can be classified in two groups. In the first group (*C. necator* H16, *P.*
287 *chlorophis* PA63, *P. montiellii* MO2, *P. putida* LS46, and *P. putida* IRN22) cell density decreased
288 dramatically between day 3 and day 10, and then stabilized (or increased very slowly) between day 10
289 and 21. In the second group (*M. luteus* IRN20, *D. tsuruhatensis* IRN27 and *A.pitti* IRN19), cell density
290 also decreased sharply between day 3 and day 10, but then increased significantly between day 10 and
291 day 21.

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292 ***Biomass production and LDPE weight loss***

293 LDPE is a carbon source that is not easily metabolized by bacteria, so large accumulations of cell
294 mass were not expected. Cell mass production on LDPE media after 21 days of incubation varied
295 between 0.15 ± 0.007 g L^{-1} CDW for *M. luteus* IRN20 and 0.37 ± 0.05 g L^{-1} for *C. necator* H16 (Fig
296 2). Degradation of LDPE also varied for each bacterium. The percent decrease in LDPE mass ranged
297 from 18.9% ± 0.072 for *M. luteus* IRN20 to 33.7% ± 0.12 for *C. necator* H16 (Figure 3). *A. pitti* IRN19
298 also appeared to be an effective bacterium for degradation LDPE particles. Although *A. pitti* IRN19
299 displayed the lowest cell mass production on LDPE rather than first bacterial group (Figure 2), it
300 reduced LDPE mass by approximately 30% (Figure 3), and generated a large range of alkane
301 hydrolysis products (Table 3). Also, although *Pseudomonas putida* LS46 produced less biomass that *C.*
302 *necator* H16 (Figure 2), there was no statistical difference in the percent decrease in LDPE mass
303 caused by *P. putida* LS 46 and *C. necator* H16 (Figure 3).

304 ***GC Analysis of material caused by LDPE biodegradation***

305 Hydrolysis products from microbial degradation of LDPE were analyzed by GC-FID
306 chromatography. Comparisons of GC chromatograms for two bacteria, *A. pitti* IRN19 and *D.*
307 *tsuruhatensis* IRN27, with alkane standard solution are seen in Figure S2. The concentrations of each
308 saturated carbon chain for the 8 bacteria tested are shown in Table 3. The data strongly suggest LDPE
309 chain biodegradation by some of the bacteria tested, and that different bacteria generate different
310 hydrolysis products. The hydrolysis products were confirmed to consist of saturated linear alkanes with
311 carbon chain lengths of C_{22} to C_{32}, because they matched perfectly with the linear alkane standards.
312 Greater than 65% of the hydrolysis products generated by all tested bacteria were C_{25} alkanes. The
313 greatest variation in hydrolysis product chain length (C_{24} to C_{32}) was observed in the culture
314 supernatants of *A. pitti* IRN19 and *D. tsuruhatensis* IRN27, which both produced a wide-range of
315 alkanes during growth on LDPE.

316 ***Scanning Electron Microscopy: Biofilm formation***

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317 All bacteria tested were observed adhering to the surface of the PE (Figure 4). *P. putida* LS46, *C.*
318 *necator* H16, and *A. pitti* IRN19 were observed adhering to the LDPE particles, and often were
319 observed in localized clumps (Figures 4B, 4C, and 4D). Overall, solid surfaces that are in contact with
320 water in the environment tend to form microbial colonization, but cell surface hydrophobicity has been
321 attributed as one of the most important factors in biofilm formation (Das and Kumar 2013), so
322 colonization of hydrophobic PE particles by cells is to be expected.

323 ***PHA accumulation and monomer composition***

324 *Cupriavidus necator* H16 is known to synthesize and accumulate the short chain length
325 polyhydroxyalkanoate (scl-PHA), poly (3-hydroybutyrate), also referred to as PHB. *P. putida* LS46 is
326 known to synthesize and accumulate medium chain length polyhydroxyalkanoates (mcl-PHAs). Both
327 species produced these polymers after culturing with LDPE particles. *Acinetobacter pitti* IRN19 was
328 also examined for PHA production after growing on LDPE particles. Table 4 shows the biomass
329 production and GC analysis of biomass for these three bacteria after 21 days of culturing in Ramsay's
330 media with 1% LDPE as the sole carbon source.

331 *Cupriavidus necator H16* generated the highest LDPE weight loss (33.75% ± 1.2), but only very
332 few alkane hydrolysis products were detected compared with other bacterial cultures: only 2.64% ±
333 0.61 (per 100 mg LDPE powder) compared with *D. tsuruhatensis* IRN27 (8.83% ± 0.09), *A. pitti*
334 IRN19 (7.53% ± 0.38), and *M. luteus* IRN20 (3.71% ± 0.11). GC analysis of *C. necator* H16 biomass
335 after 21 days of culture with LDPE displayed three peaks (6.33, 7.08 and 7.7 minutes retention time
336 after injection), which corresponded to the methyl esters of 3-hydroxybutanoic acid (C4), 3-
337 hydroxyvaleric acid (C5), and benzoic acid (the internal standard). The molar ratio of the subunit
338 composition was 94.9 ± 0.6% C4 and 5.03 ± 0.56% C5 (Figure S3). No statistically significant
339 differences in biomass production (in Ramsay's media plus 1% LDPE) were observed between *P.*
340 *putida* LS46 and *A. pitti* IRN19, and both bacteria generated the same PHA composition, which

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341 consisted of 3-hydroxyhexanoic methyl ester (C6), 3-hydroxyoctanoic ester (C8), 3-hydroxydecanoic
342 ester (C10), 3-hydroxydodecanoic ester (C12) and 3-hydroxytetradecanoic ester (C14), with
343 approximately half of PHA composition consisting of the C6 subunit.

344 **Discussion**

345 While most early studies of microbial degradation of polyethylene used polyethylene that had been
346 subjected to some form of pretreatment, several recent studies have indicated microbial degradation of
347 untreated polyethylene (Kyaw et al. 2012; Yoon et al. 2012; Peixoto et al. 2017). Our results are
348 consistent with these reports and further show that in addition to cell mass production, some bacteria
349 were also able to synthesize and accumulate PHA polymers after growth on LDPE.

350 The growth curves suggest that during incubation, two types of carbon sources were consumed.
351 LDPE is generally amorphous, with short branches (10-30 CH_3 per 1000 C atoms), consisting of one or
352 more co-monomers, such as 1-butene, 1-hexene, and 1-octene. This branching system prevents the PE
353 molecules from stacking closely together, making the LDPE chains more accessible. Theus, the tertiary
354 carbon atoms that are present at the branch sites are more susceptible to attack. Also, some structural
355 variations, such as unsaturated carbon-carbon double bonds, carbonyl-groups, and hydroperoxide-
356 groups, formed during polymerization and subsequent processing, may also be present in the PE
357 polymers (Ojeda et al. 2011). These short side chains may be consumed first by the bacteria,
358 accounting for the rapid growth in the first three days.

359 Statistical analysis showed a positive relationship between bacterial biomass production and LDPE
360 weight loss, but varied between weak and strong among the bacteria tested, suggesting that the loss in
361 LDPE mass may be more a function of the type and/or amount of enzyme(s) secreted, rather than total
362 numbers of cells.we also observed greater than 65% of the hydrolysis products generated by all tested
363 bacteria were C_{25} alkanes. Eyheraguibel et al. (2017) have shown that the molecular weight of
364 extracted oligomers was lower than 850 Da, with a maximum chain length of 55 carbon atoms, and that
365 strong shift to smaller molecules (< 450 Da, 25 carbon atoms) was observed, suggesting that longer

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366 molecules disappeared more rapidly than the smaller ones. This has provided a new perspective on
367 biodegradation processes, suggesting that extracellular mechanisms leading to chain cleavage may play
368 a significant role in polyethylene biodegradation (Eyheraguibel et al. 2017). In our study, there were
369 also some small peaks (Figure S2), other than alkanes, detected by GC in the culture superntants, but
370 these were not identified. Thus, other reaction products may have been present. However, the focus of
371 this study was not to specifically analyze the mechanims of LDPE biodegradation. This is the objective
372 of a future study.

373 In a recent study, Guzik et al. (2014) reported growth and PHA accumulation of *Acinetobacter*
374 *calcuaceticus* (0.24 0.1 g l^{-1} cdw; 4.1 0.1 % cdw, respectively) and *Pseudomonas aeruginosa* (0.39 0.4
375 g l^{-1} cdw; 18.9 0.7 % cdw, respectively), on 2% (w/v) PE pyrolysis wax as the sole carbon source over
376 48 h. In these experiments, PE was subjected to thermal pre-treatment in the absence of oxygen
377 (pyrolysis) at very high temperatures (300 ^{o}C to 500 ^{o}C), which generated a complex mixture of low
378 molecular weight paraffins with carbon chain lengths from C8 to C32 (PE pyrolysis wax) (Guzik et al.
379 2014). Thus, Guzik et al. demonstrated that *A. calcuaceticus* and *P. aeruginosa* were able to metabolize
380 alkane hydrocarbons, but did not demonstrate the direct catabolism of LDPE. Our work differs from
381 that of Guzik et al. as it demonstrates direct utilization of LDPE as a sole carbon and energy source and
382 its bioconversion to PHAs polymers.

383 We have demonstrated that several bacteria can utilize LDPE powder as a sole carbon source for
384 growth and that PE hydrolysis products, detected in the culture supernatants, were generated during cell
385 growth, strongly suggesting that biofragmentation had occurred. As LDPE was the sole carbon source,
386 to by deduction, we can state that the cell growth and PHA production was a consequence of LDPE
387 metatabolism. Although the maximum degradation of LDPE observed was 33.7%, we have
388 demonstrated that *C. necator* H16, *P. putida* LS46, and *A. pitti* IRN19 could assimilate LDPE, could
389 not only produce biomass, but could also generate alkane hydrolysis products, and accumulate
390 biopolymers in the form of the short and medium chain length polyhydroxyalkanoates. Thus, our data

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391 provide strong evidence of polyethylene biofragmentation and bioassimilation. However, the purpose
392 of the current manuscript was to report on our initial introduction for untreated LDPE degrading
393 bacteria and more details studies are underway and will be reported in a future publication.

394 The potential for degradation of polyethylene by microorganisms is widely accepted, but our
395 understanding of the mechanisms of microbial degradation, as well as the enzymes and corresponding
396 genes involved is very limited (Usha et al. 2011). The application of genome sciences and molecular
397 biology may help clarify these mechanisms and improve the biodegradation of LDPE and other
398 hydrocarbon compound contaminants in the environment. Yoon et al (2012) investigated
399 biodegradation of non-oxidized low molecular weight polyethylene (LMWPE), whose molecular mass
400 was was well above the upper limit that can penetrate microbial membranes. Using *Pseudomonas sp*.
401 E4, isolated from soil contaminated with crude oil, and a recombinat *Escherichia coli* BL21that
402 expressed the alkane hydroxylase gene (alkB) from *Pseudomonas sp*. E4, Yoon et al (2012) showed
403 that the AlkB enzyme played a central role in LMWPE degradation, even in the absence of the other
404 specific enzymes like rubredoxin and rubredoxin reductase. Jeon and Kim (2016) compared the
405 functional characterization of alkane monooxygenases for LMWPE biodegradation, and showed that
406 the AlkB2 enzyme was more effective in degrading LMWPE than the AlkB1 enzyme, and that the
407 regulation mechanism of AlkB2 was different from that of AlkB1.

408 Although some researchers have reported that that the alkane catabolic pathway flows from β-
409 oxidation to the TCA cycle (Yoon et al. 2012), Eyherraguible et al. (2017) suggested that extracellular
410 mechanisms leading to enzymatic oxidation and chain cleavage of PE polymers are also important.
411 These insights into the mechanisms of microbial degradation of PE provide a new prespective on
412 biodegradation processes which must be further explored.

413 **Acknowledgements**

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537 **Figure Captions**

538

539 **Figure 1.** Growth curves from optical density for eight LDPE-degrading bacteria over 21 days.

540

541 **Figure 2.** Cell mass production (g L^{-1}) by different bacteria cultured with LDPE at day 21. Statistical
542 analyses showed that each treatment sample had a skewed distribution and was non-parametric. Boxes
543 show the upper and lower quartiles: the solid lines in the boxes represent the median of the sample; the
544 dotted lines represent standard deviations (i.e. the largest and smallest values). Differences in means
545 were indicated using lowercase letters (a, b, c, d, and e). Treatments with the same letter were not
546 statistically different ($p < 0.05$).

547

548 **Figure 3.** Percent decrease in LDPE mass in bacterial cultures at day 21. Statistical analyses showed
549 that each treatment sample had a skewed distribution and was non-parametric. Boxes show the upper
550 and lower quartiles: the solid lines in the boxes represent the median of the sample; the dotted lines
551 represent standard deviations (i.e. the largest and smallest values). Differences in means were indicated
552 using lowercase letters (a, b, c, d, and e). Treatments with the same letter were not statistically different
553 ($p < 0.05$).

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554

555 **Figure 4.** Scanning electron micrographs (magnification × 2,0000) of A) the LDPE surface with no

556 microbial treatment; B) microbial colonization by *Pseudomonas putida* LS46; C) microbial

557 colonization by *Cupriavidus necator* H16; and D) microbial colonization by *Acinetobacter pitti IRN19.*

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1 **Tables**

2

3 **Table 1.** Peak definition and wave length position (cm^{-1}) at different points of the curves (Baselines,

4 edge and peak).

	Peak position	**Left Baseline**	**Left Edge**	**Right Edge**	**Right Baseline**
CH2 asymmetric (CHSii)	2914.72	2983.339	2929.657	2896.586	2793.48
CH2 symmetric (CHSi)	2847.885	2999.692	2857.678	2838.224	2770.135
C=C	1462.559	1490.705	1473.66	1456.785	1425.211
Carbonyl	1701.121	1736.244	1715.731	1696.193	1669.237

5

6 **Table 2.** Data from control experiments.

Control	Inoculation	Culture media	Results (OD)
Chemical contamination	*E. coli* BL DE3	1% LDPE, 0.1% Tween 80, plus minimal salt media	0.09 ± 0.016
Microbial contamination	No inoculant	1% LDPE, 0.1% Tween 80, plus minimal salt media	negative
Other carbon source consumption	Each isolate tested	No LDPE, 0.1% Tween 80, plus minimal salt media	0.05 ± 0.008

7
8 * Average of optical density from each bacterial growth after 3 days. Maximum OD level during the
9 incubation time.

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Table 3. Alkane hydrolysis products detected after microbial biodegradation of LDPE by different bacteria, after 21days of incubation.

Alkanes	**C_{22}**	**C_{23}**	**C_{24}**	**C_{25}**	**C_{26}**	**C_{27}**	**C_{28}**	**C_{29}**	**C_{30}**	**C_{31}**	**C_{32}**	**Total**
D. tsuruhatensis IRN27	0.32 ± 0.03 [1]	0.09 ± 0.02	0.24 ± 0.03	7.26 ± 0.23	0.42 ± 0.05	0.35 ± 0.001	0.15 ± 0.06	0	0	0	0	8.83 ± 0.09[a]
A. pitti IRN19	0	0	0.10 ± 0.02	5.15 ± 0.34	0.44 ± 0.05	0.50 ± 0.02	0.47 ± 0.03	0.31 ± 0.01	0.33 ± 0.17	0.22 ± 0.01	Nd	7.53 ± 0.38[b]
M. luteus IRN20	1.11 ± 0.02	Nd	Nd	2.44 ± 0.13	0.10 ± 0.02	0.07 ± 0.02	0	0	0	0	0	3.71 ± 0.11[c]
C. necator H16	0	0	Nd	2.55 ± 0.579	0.08 ± 0.030	0	0	0	0	0	0	2.64 ± 0.61[d]
P. monteilii MO2	0.42 ± 0.01	0	0	0	0	0	0	0	0	0	0	0.42 ± 0.01[e]
P. putida LS46	0	0	0	0	0	0	0	0	0	0	0	0
P.putida IRN22	0	0	0	0	0	0	0	0	0	0	0	0
P. chlorophis	0	0	0	0	0	0	0	0	0	0	0	0

Nd: Not detected, because of the presence of a peak that was too small to quantify; [1] Concentrations of alkanes detected are expressed in mg per 100 mg PE powder added initially to the culture media; Superscripts (a, b, c, d, and e) indicate results of statistical analyses, with different letters indicating significant differences at $p \leq 0.05$.

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14 **Table 4.** Biomass production, PHA accumulation, and mol% subunit composition in *C. necator* H16,

15 *P. putida* LS46, and *A. pitti IRN19* after 21 days of culture in Ramsay's media with 1% LDPE.

Species:	***C. necator* H16**	***P. putida* LS46**	***A. pitti IRN19***
cdw (g L^{-1}):	0.34 ± 0.02[a]	0.24 ± 0.01 [b]	0.23 ± 0.05 [b]
PHA accumulation (% cdw):	3.18 ± 0.4	0.54 ± 0.037	0.49 ± 0.019
Monomer composition	**Mol%**	**Mol%**	**Mol%**
C4	94.90 ± 0.61	0	0
C5	5.03 ± 0.56	0	0
C6	0	58.60 ± 0.48	50.50 ± 1.34
C8	0	16.86 ± 3.58	0.53 ± 0.25
C10	0	8.49 ± 0.16	34.25 ± 0.95
C12	0	7.35 ± 0.30	9.19 ± 0.44
C14	0	8.64 ± 2.63	11.47 ± 0.32

16

17 [a,b] Different lower case letters (a, b) indicate significant difference ($p < 0.05$).

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18 **Figure Captions**

19

20 **Fig 1.** Growth curves from optical density for eight LDPE-degrading bacteria over 21 days.

21

22 **Figure 2.** Cell mass production (g L^{-1}) by different bacteria cultured with LDPE at day 21. Statistical
23 analyses showed that each treatment sample had a skewed distribution and was non-parametric. Boxes
24 show the upper and lower quartiles: the solid lines in the boxes represent the median of the sample; the
25 dotted lines represent standard deviations (i.e. the largest and smallest values). Differences in means
26 were indicated using lowercase letters (a, b, c, d, and e). Treatments with the same letter were not
27 statistically different ($p < 0.05$).

28

29 **Figure 3.** Percent decrease in LDPE mass in bacterial cultures at day 21. Statistical analyses showed
30 that each treatment sample had a skewed distribution and was non-parametric. Boxes show the upper
31 and lower quartiles: the solid lines in the boxes represent the median of the sample; the dotted lines
32 represent standard deviations (i.e. the largest and smallest values). Differences in means were indicated
33 using lowercase letters (a, b, c, d, and e). Treatments with the same letter were not statistically different
34 ($p < 0.05$).

35

36 **Figure 4.** Scanning electron micrographs (magnification × 2,0000) of A) the LDPE surface with no
37 microbial treatment; B) microbial colonization by *Pseudomonas putida* LS46; C) microbial
38 colonization by *Cupriavidus necator* H16; and D) microbial colonization by *Acinetobacter pitti IRN19.*

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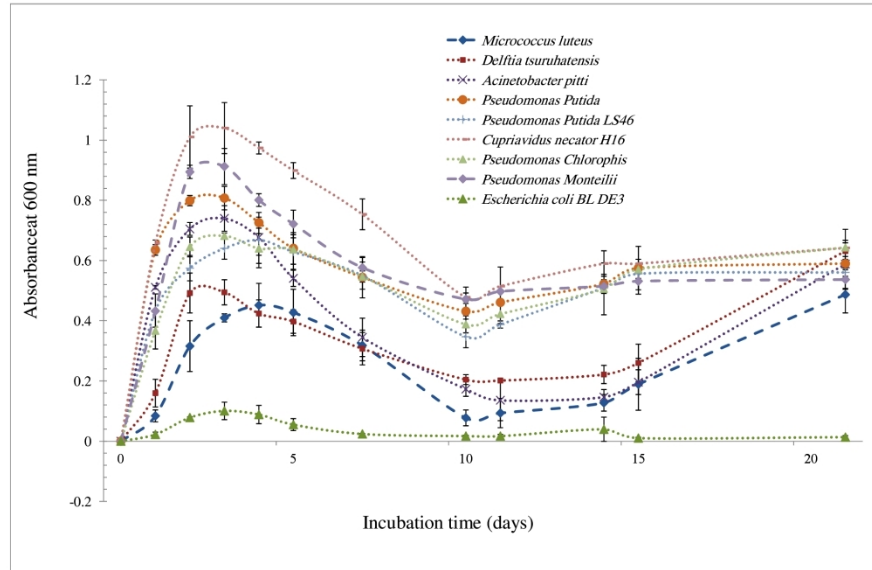

Figure 1. Growth curves from optical density for eight LDPE-degrading bacteria over 21 days.

197x139mm (300 x 300 DPI)

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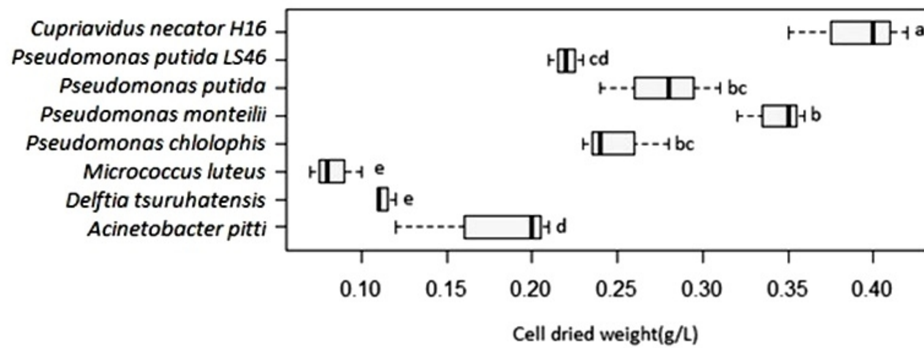

Figure 2. Cell mass production (g L-1) by different bacteria cultured with LDPE at day 21. Statistical analyses showed that each treatment sample had a skewed distribution and was non-parametric. Boxes show the upper and lower quartiles: the solid lines in the boxes represent the median of the sample; the dotted lines represent standard deviations (i.e. the largest and smallest values). Differences in means were indicated using lowercase letters (a, b, c, d, and e). Treatments with the same letter were not statistically different ($p < 0.05$).

69x27mm (300 x 300 DPI)

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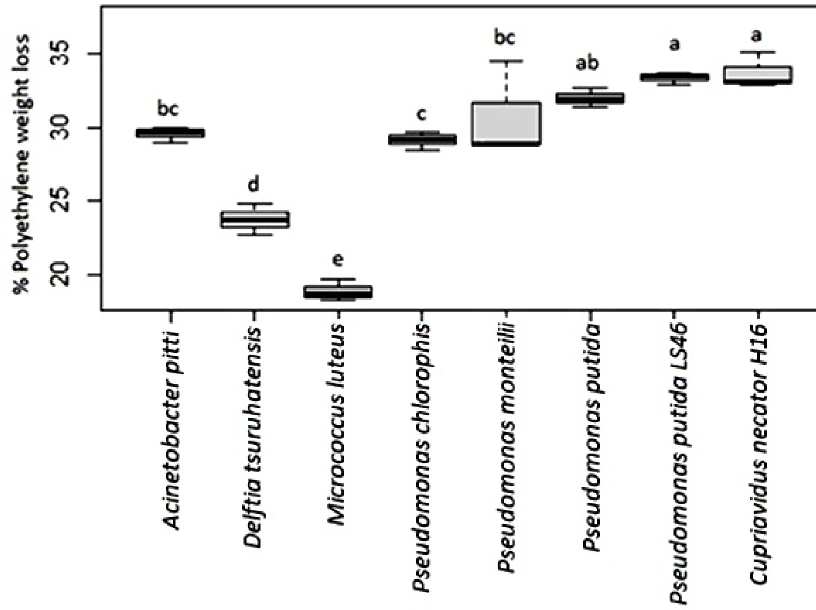

Figure 3. Percent decrease in LDPE mass in bacterial cultures at day 21. Statistical analyses showed that each treatment sample had a skewed distribution and was non-parametric. Boxes show the upper and lower quartiles: the solid lines in the boxes represent the median of the sample; the dotted lines represent standard deviations (i.e. the largest and smallest values). Differences in means were indicated using lowercase letters (a, b, c, d, and e). Treatments with the same letter were not statistically different ($p < 0.05$).

59x39mm (300 x 300 DPI)

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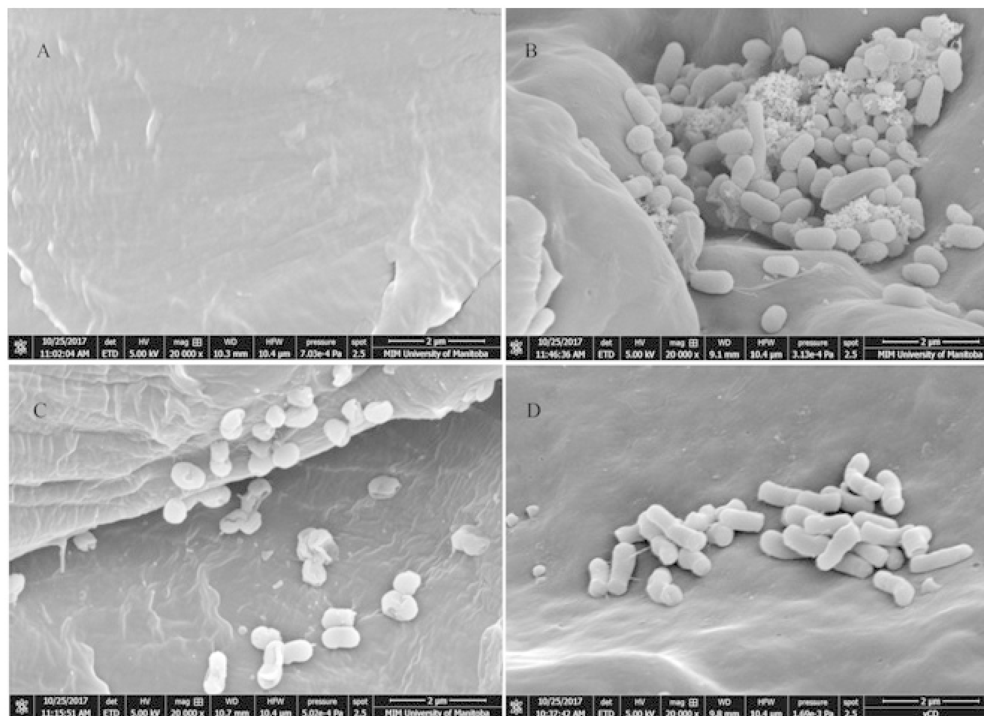

Figure 4. Scanning electron micrographs (magnification × 2,0000) of A) the LDPE surface with no microbial treatment; B) microbial colonization by Pseudomonas putida LS46; C) microbial colonization by Cupriavidus necator H16; and D) microbial colonization by Acinetobacter pitti IRN19.

56x40mm (300 x 300 DPI)