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1 **Aerobic biotransformation of the antibiotic ciprofloxacin by *Bradyrhizobium* sp. isolated**

2 **from activated sludge**

3 Accepted manuscript

4 **Chemosphere**

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23 **Abstract**

24 Ciprofloxacin (CIP) is an antibiotic that is widely used to treat bacterial infections and is poorly
25 biodegraded in the wastewater treatment process. In this study, a CIP-degrading strain
26 (GLC_01) was successfully retrieved from activated sludge by enrichment and isolation. The
27 obtained bacterial strain shares over 99% nucleotide identity of the 16S rRNA gene with
28 *Bradyrhizobium* spp. Results show that *Bradyrhizobium* sp. GLC_01 degraded CIP via
29 cometabolism with another carbon substrate following a first-order kinetics degradation
30 reaction. CIP degradation by *Bradyrhizobium* sp. GLC_01 increased when the concentration
31 of the primary carbon source increased. The biodegradability of the primary carbon source also
32 affected CIP degradation. The use of glucose and sodium acetate (i.e. readily biodegradable),
33 respectively, as a primary carbon source enhanced CIP biotransformation, compared to starch
34 (i.e. relatively slowly biodegradable). CIP degradation decreased with the increase of the initial
35 CIP concentration. Over 70% CIP biotransformation was achieved at 0.05 mg L^{-1} whereas CIP
36 degradation decreased to 26% at 10 mg L^{-1}. The phylogenetic identification and experimental
37 verification of this CIP-degrading bacterium can lead to a bioengineering approach to manage
38 antibiotics and possibly other persistent organic contaminants during wastewater treatment.

39 **Key words**: Ciprofloxacin (CIP); *Bradyrhizobium*; biotransformation; cometabolism.

40 **1. Introduction**

41 The occurrence of trace organic contaminants (TrOCs) including pharmaceuticals, personal
42 care products, steroid hormones, and industrial chemicals in sewage and sewage-impacted
43 water bodies is of considerable human health and ecological concern. Some of these
44 compounds, such as pharmaceuticals and personal care products, are indispensable in our
45 modern society. Others, such as steroid hormones, are naturally and excreted continuously by
46 mammals including human beings, and thus their release is unavoidable. There is a growing
47 concern that the occurrence of TrOCs in the environment can affect aquatic ecology due to
48 their biologically active properties (Clara et al., 2012; Dong et al., 2015; Luo et al., 2014; Tran
49 et al., 2018). Another notable effect is the spread and proliferation of microbes that are
50 persistent to antibiotics in the environment (Halling-Sørensen et al., 2000; Martínez, 2008).

51 Antibiotics are widely used in medicine and agriculture. However, only a small portion can be
52 metabolised by humans and animals, and the rest is released into the environment (Nguyen et
53 al., 2017). As an example, ciprofloxacin (CIP) is commonly used to treat bacterial infection
54 and is frequently detected at elevated concentration in secondary effluent and hospital
55 wastewater (ca. 10 – 200 μg L^{-1}) and pharmaceutical manufacturing wastewater (ca. 6.5 – 31
56 mg L^{-1}) (Larsson et al., 2007; Nguyen et al., 2017; Tran et al., 2018). Indeed, CIP
57 concentrations in some of these wastewaters exceed the predicted no-effect concentrations for
58 several aquatic organisms (Robinson et al., 2005). CIP has also been suspected to cause the
59 development and transmission of antibiotic resistance genes in environmental microbiota
60 (Martínez, 2008; Turolla et al., 2018; Zhang et al., 2013).

61 Biological treatment plays a crucial role in the removal of TrOCs prior to effluent discharge
62 into the environment (Luo et al., 2014). Concerted research efforts in recent years have
63 significantly improved our understanding of the biodegradation of TrOCs by biological
64 (including both aerobic and anaerobic) treatment. For example, it has been established that

biodegradation of TrOCs is governed by their physicochemical properties, especially the presence of either electron-withdrawing or donating functional groups in their molecular structure (Tadkaew et al., 2011; Wijekoon et al., 2015). TrOCs with electron-withdrawing functional groups are expected to be poorly removed (i.e. < 20%) while those with electron-donating functional groups are expected to be well removed (i.e. > 70%) by activated sludge treatment (Tadkaew et al., 2011). Based on this theory, Tadkaew et al. (2011) has developed a qualitative framework for the prediction of TrOC removal by activated sludge treatment.

Although the qualitative prediction framework proposed by Tadkaew et al. (2011) has been successfully validated by other authors (Li et al., 2015; Naghdi et al., 2018; Tran et al., 2018), it has not yet been able to account for occasionally peculiar and unusually high removal values of persistent TrOCs reported in the literature. Indeed, negligible removal efficiency (<15%) of CIP by activated sludge treatment has been widely reported (Jia et al., 2012; Li & Zhang, 2010; Lindberg et al., 2006) possibly due to the presence of fluoro which is a strong electron-withdrawing functional group in its molecular structure. On the other hand, CIP removal as high as 52.8% by a laboratory-scale membrane bioreactor has been reported by (Dorival-García et al., 2013). Recent research suggests that these occasionally and unusually high removal values of persistent TrOCs by biological treatment might be attributed to the microbial composition of the biomass (Vuono et al., 2016). In other words, there are rare microbial strains that can effectively metabolise otherwise poorly biodegradable TrOCs. The identification of these microbial strains and elucidation of their metabolic pathways can provide new insights into a bioaugmentation approach for the treatment of persistent TrOCs.

Although CIP is poorly biodegradable, a few CIP-degrading strains have been reported. A fluorobenzene-degrading bacterium *Labrys portucalensis* F11 could substitute the fluoride group in CIP with a hydroxyl group. This strain was isolated from an industrially contaminated site, however, the site characteristics were not provided (Amorim et al., 2014). Another CIP-

90 degrading strain *Thermus* sp. was isolated via a serial enrichment of pharmaceutical sludge
91 with CIP concentration of 1, 5 and 20 mg L^{-1} (Pan et al., 2018). This strain was a thermophilic
92 microbe (70 °C), making it difficult to apply in wastewater treatment which commonly operate
93 at 20 to 30 °C (Pan et al., 2018). Freshwater microalgae *Chlamydomonas mexicana* showed
94 13% removal of CIP after 11 days of cultivation (Xiong et al., 2017). A mixture of anaerobic
95 sulfate-reducing bacteria showed moderate degree of CIP biodegradation (Jia et al., 2018). Liao
96 et al. (2016) reported that activated sludge could harbour CIP-degrading strains in the classes
97 of *Gammaproteobacteria*, *Bacteroidia* and *Betaproteobacteria*. Identifying CIP-degrading
98 strains from activated sludge is an important step towards the improvement of CIP removal.

99 Previous studies have demonstrated that long-term exposure of activated sludge microbiome
100 to TrOCs can alter the microbial community and in some cases selectively enrich specific
101 microbes with enhanced affinity for TrOCs biodegradation (Moreira et al., 2014; Navaratna et
102 al., 2012; Qu & Spain, 2010; Terzic et al., 2018; Zhou et al., 2013). An early example was
103 observed for acesulfame (ACE), a synthetic sweetener. ACE was reportedly persistent to
104 biological degradation in German WWTPs with less than 5% removal in 2010 (Kahl et al.,
105 2018) when it was first introduced in the market. Over time, the activated sludge microbial
106 community seemingly evolved to biotransform ACE. Recently, more than 85% ACE removal
107 by conventional wastewater treatment has been reported (Kahl et al., 2018). Exposure of
108 activated sludge microbiome to TrOCs (at a level that is higher than the environmentally
109 relevant concentration) could increase selective pressure and shorten the evolution time. The
110 initial activated sludge was unable to degrade macrolide antibiotics at concentration of 1 – 10
111 mg L^{-1}. After two months of exposure at 10 mg L^{-1}, the removal efficiency was increased to
112 99% (Terzic et al., 2018). Accordingly, TrOC-degrading strains have been identified for the
113 removal of previously reported persistent compounds (Moreira et al., 2014; Mulla et al., 2016;
114 Pan et al., 2018; Yu et al., 2007).

115 This study aims to retrieve CIP-degrading strains from activated sludge and subsequently
116 characterise the degradation of CIP by the strains. The strains are obtained using enrichment
117 and isolation methods, and then further characterised in terms of their genotypes using 16S
118 rRNA gene-based sequencing. Phenotypes of the strains are characterised against a number of
119 abiotic factors (i.e. CIP concentrations, concentration and types of primary carbon sources).
120 CIP removal mechanisms (i.e. abiotic, adsorption and biodegradation) are evaluated. By
121 identifying and comprehensively examining CIP-degrading strains from activated sludge, this
122 study provides new insights that can be used to enhance the removal persistent TrOCs by
123 biological treatment.

2. Materials and methods

124 **2.1 Chemicals**

125 Analytical grade (> 98% purity) of ciprofloxacin hydrochloride monohydrate was purchased
126 from Sigma-Aldrich (Singapore). A stock solution containing 1 g L^{-1} was prepared in Milli-Q
127 water for all subsequent experiments. A growth medium containing glucose (1.8 g L^{-1}), urea
128 (35 mg L^{-1}), KH_2PO_4 (17.5 mg L^{-1}), $MgSO_4$ (17.5 mg L^{-1}), and $FeSO_4$ (10 mg L^{-1}) was
129 prepared following a procedure previously described by Oh et al. (2013). R2A agar was
130 purchased from (DB Diagnostics, Singapore).

131 **2.2 Enrichment protocol**

132 Three identical laboratory scale reactors (0.9 L active volume each) were seeded with activated
133 sludge from an aeration tank of wastewater treatment plant in Singapore. The reactors were
134 aerated to achieve a dissolved oxygen content of 3 mg L^{-1} and maintained at 22.5 ± 0.5 °C. The
135 reactors were fed every 3.5 days by withdrawing 0.3 L mixed liquor and replacing with a
136 freshly-prepared growth medium, resulting in 10.5 days of retention time. Soluble chemical
137 oxygen demand (sCOD) removal and volatile suspended solids (VSS) were monitored every

138 3.5 days and the system achieved a steady period with sCOD removal >90% and VSS 0.98 ±

139 0.2 g L^{-1} after 30 days.

140 The reactors were then fed with growth medium containing 5 mg L^{-1} CIP for 4 months to

141 encourage the proliferation of CIP degrading bacterial strains. The sludge was then obtained

142 from these reactors, mixed together into an inoculum source, and incubated on agar plate (R2A

143 agar) supplementing with 5 mg L^{-1} CIP. The derived colonies were subsequently transferred to

144 another agar plate four times by repeated streaking culture until a single colony was confirmed.

145 This enrichment and isolation procedure resulted in two separated colonies (designated as

146 strain GLC_01 and GLC_02). They were then evaluated for CIP removal. Strain GLC_01

147 demonstrated CIP removal capacity and was selected for future experiments. A single colony

148 of strain GLC_01 was suspended into 50 mL growth medium containing CIP and incubated on

149 a rotary shaker at 25 °C and 150 rpm. This culture was kept as the inoculum source for genotype

150 and phenotype characterised experiments.

151 **2.3 Evaluation of CIP removal routes**

152 The potential removal routes of CIP including biotransformation (experiment I), adsorption

153 (experiment II), and utilization of CIP as sole carbon source (experiment III) by the strain

154 GLC_01 were elucidated by batch experiments. In experiment I, the growth medium was

155 inoculated with active GLC_01 cells at the initial $OD_{620\ nm}$ of 0.1 that was equivalent to 10^5

156 colony forming unit (CFU/mL). In experiment II, inactive (heat-killed) GLC_01 cells were

157 added to get an $OD_{620\ nm}$ of 1.6 ± 0.01. This value was pre-determined to maintain the same

158 level of cell biomass in experiment I and II, and thus adsorption of CIP would be comparable

159 in these two experiments. In experiment III, the growth medium was inoculated with same

160 amount of active GLC_01 cells as in experiment I but did not have any glucose - which was

161 the primary carbon source. All experiments were prepared with 50 mL growth medium into

162 250 mL-sterile flasks. Experiment IV was prepared without GLC_01 cells to determine the

163 removal of CIP by abiotic factors (i.e. photolysis, hydrolysis and volatilization). CIP was added
164 in all experiments at concentration of 4.89 ± 0.01 mg L^{-1} (*n*= *12*). All experimental flasks were
165 covered and incubated on a rotary shaker at 25 ˚C and 150 rpm. Samples were collected at
166 interval time of 1 day for 8 consecutive-days. All laboratory apparatuses were autoclaved at
167 121˚C and 15 min to avoid any contamination. Residues of CIP in each experiment were
168 measured using a HPLC method (see Section 2.5.2). COD removal was measured in
169 experiment I (active cells). Cell growth rate in experiment I, II and III was measured using
170 methods as described in Section 2.5.1.

171 **2.4 Influence of abiotic factors on CIP removal**

172 To evaluate the variance in influence of initial COD concentrations, a range of COD
173 concentration (i.e. 150, 500, 1000 and 2000 mg L^{-1}) was used. Glucose was used as the primary
174 carbon source with different amounts that were equivalent to the desired COD levels. All
175 experimental flasks were covered and incubated on a rotary shaker at 25˚C and 150 rpm.
176 Samples were collected at interval time of 1 day for 8 consecutive-days. Other experimental
177 conditions were maintained as in experiment I (see Section 2.3).

178 Influence of primary carbon sources on the performance of strain GLC_01 was elucidated in
179 this study. Glucose, sodium acetate and starch were selected to have diverse chemical structures
180 and biodegradable levels. Each carbon source was prepared to achieve 2000 mg L^{-1} initial
181 COD. Other experimental conditions were maintained as in experiment I (see Section 2.3).

182 Impact of initial CIP concentrations on the performance of strain GLC_01 was investigated.
183 The concentration of CIP varied from 0.05 to 10 mg L^{-1}. This range was selected to represent
184 environmentally relevant concentrations and occasionally high concentrations (e.g. in
185 pharmaceutical manufacturing wastewater) (Halling-Sørensen et al., 2000; Jia et al., 2012;
186 Larsson et al., 2007). Glucose was used as the primary carbon source in this experiment at a

187 concentration of 2000 mg L^{-1} (as expressed by COD). Other experimental conditions were
188 maintained as in experiment I (see Section 2.3).

189

190 **2.5 Analytical methods**

191 **2.5.1 COD and microbial growth**

192 COD concentration was measured by using digestion vials (Hach, Singapore) and Hach
193 DR3900 spectrophotometer following the manufacturer's instruction. Two analytical ranges
194 (20 – 1500 mg L^{-1}) and (0 – 150 mg L^{-1} COD) were used for initial and after treatment samples,
195 respectively. VSS in the reactors was measured following the standard method 2540A. Cell
196 growth was quantified by optical density (OD) at 620 nm and was measured using a Shimadzu
197 DR 6000 spectrophotometer.

198 **2.5.2 CIP concentration**

199 CIP concentration was measured by a Shimadzu HPLC system equipped with Shim-Pack GIST
200 Phenyl, 5 µm, 4.6 x 250 mm column (Shimadzu Asia Pacific Pte Ltd). The detection
201 wavelength and sample injection volume were 280 nm and 100 µL, respectively. The mobile
202 phase comprised of 60% acetonitrile and 40% Milli-Q water buffered with 25 mM NaH_2PO_4
203 at pH 2.5. The mobile phase was delivered in an isocratic elution mode, at 1.8 mL/min through
204 the column for 3.5 min. The limit of quantification for CIP using these conditions was
205 approximately 10 µg L^{-1}. CIP removal was calculated using the following equation: Removal
206 (%) = $[(C_0 - C_t) \times 100] \div C_0$, where C_0 and C_t denote the concentration of CIP at day 0 and t,
207 respectively. Statistical testing for differential CIP removal by different experiments was
208 conducted using the Student's t-test in Excel.

209 A LC-MS/MS system (Agilent 6400 Series Triple Quadrupole LC/MS-MS) was used to detect
210 any by-products from the treatment. Electrospray ionisation (ESI) source is applied technique.
211 To improve MS outcomes, several preliminary experiments were conducted to optimize the

212 LC-MS parameters. The target components were separated on a C18 column (particle size 1.5
213 μm, ID 2.1 μm, L 10 cm). Two eluents, A (acetonitrile + 0.1% (v/v) formic acid) and B (water
214 + 0.1% (v/v) formic acid) were delivered at 0.2 mL min^{-1} through the column for 11 min in the
215 following time-dependent gradient proportions: [Time (min), % of B] = [0, 90], [1, 90], [6,
216 10], [7, 10], [7.1, 90], [10, 90]. The column temperature was maintained at 35˚C. The mass
217 spectrometric data were collected from 100 to m/z 1000 in positive and negative ion mode. The
218 cone voltage for each sample was optimised in both positive and negative ion mode. Additional
219 detector parameters were held constant for all samples: interface temperature 350 °C;
220 nebulizing gas flow 1.5 L min^{-1}; dry gas flow 3 L min^{-1}; DL temperature 250 °C and heating
221 block 200 °C.

222 **2.5.3 DNA sequencing**

223 A single colony of the GLC_01 strain was obtained from the agar plate and cultured in 50 mL
224 growth medium supplemented with CIP on rotary shaker for 3 days, 25 °C and 150 rpm. The
225 culture was collected into sterile centrifuge tube and centrifuged at 6000 rpm for 5 min.
226 Bacterial pellet was collected and subjected to DNA extraction (MoBio PowerSoil® DNA
227 isolation kit -MOBIO, Carlsbad, CA, USA) following the manufacture's instruction. Then
228 polymerase chain reaction (PCR) was conducted using 1 μL of 10 pM/mL each of universal
229 primer (27F, *5′ AGAGTTTGATCMTGGCTCAG 3′* and 1492R, *5′*
230 *TACGGYTACCTTGTTACGACTT 3′*) (Lane, 1991), a pre-mixed solution (dNTP, buffer, Taq
231 polymerase and dye), 1 μL of extracted DNA and 18 μL of Milli-Q water into 50 μL strip cap
232 tubes. A thermocycler (Eppendorf Mastercycler) was programmed time-dependent gradient
233 proportions: pre-heating [95˚C, 5 min], denaturation [95˚C, 30 second], annealing [55˚C, 30
234 second], extension [72˚C, 1.5 min], repeat 30 cycles, additional extension [72˚C, 7 min], and
235 hold [4˚C, ∞ min]. PCR products were purified by an Ultraclean PCR clean up DNA

236 purification kit (Mo Bio Laboratories, USA). Finally, PCR products were confirmed by DNA
237 electrophoresis. The gene sequence was conducted by 1BASE Asia (Singapore).
238 The 16S rRNA sequence was submitted to the NCBI BLAST database (National Center for
239 Biotechnology Information http://www.ncbi.nlm.nih.gov/BLAST/ and run with 16S ribosomal
240 RNA (Bacteria and Archaea) to identify close relatives with strain GLC_01. The 16S rRNA
241 gene sequences of 11 species (99% nucleotide identity) and one outlier were used to construct
242 the phylogenetic tree in MEGA 7.0 with a maximum likelihood method (Kumar et al., 2016).
243 The 16S rRNA gene sequence of strain GLC_01 was deposited in GenBank under the accession
244 number of MH297488.

245 **3. Results and discussion**

246 **3.1 Identification of a CIP-degrading bacteria**

247 Two bacterial strains were retrieved by enrichment and isolation (Section 2.2) from activated
248 sludge continuously exposing to 5 mg L^{-1} of CIP in the feed. However, only strain GLC_01
249 showed the ability to degrade CIP in a growth medium. The DNA sequencing of the 16S rRNA
250 gene (1326 bp) showed that strain GLC_01 shares over 99% nucleotide identity with the genus
251 *Bradyrhizobium* (Fig. 1). This strain was classified as *Bradyrhizobium* sp. strain GLC_01.
252 Species of the genus *Bradyrhizobium* have been isolated from soil, contaminated site, drinking
253 water filtration system (e.g. sand filter, granular activated carbon column) (Hayashi et al., 2016;
254 Oh et al., 2018; Sudtachat et al., 2009).

255 Members of the genus *Bradyrhizobium* are aerobic microbes and are diverse in biochemical
256 functions such as nitrification, sulphur oxidation and aromatic degradation (Hayashi et al.,
257 2016; Oh et al., 2018; Sudtachat et al., 2009). The *Bradyrhizobium* sp. strain GLC_01 could
258 not grow under anaerobic conditions, and thus it is an aerobic microbe. A number of genus
259 *Bradyrhizobium* has demonstrated the ability to biotransform aromatic compounds. For

260 example, *Bradyrhizobium japonicum* has multiple gene copies for aromatic degradation in its
261 genome. This strain could degrade vanillate for energy and carbon source (Sudtachat et al.,
262 2009). *Bradyrhizobium* sp. strain JS329 has been successfully isolated from soil supplement
263 with 5-Nitroanthranilic acid, and it expressed a number of enzymes such as dioxygenase,
264 deaminase that cleaved the benzene ring of 5-Nitroanthranilic acid (Qu & Spain, 2010). The
265 soybean root *Bradyrhizobium elkanii* USDA94 encodes tfdAα and cadABC gene cluster,
266 which have been reported as degrading genes for herbicides like 2,4-dichlorophenoxyacetic
267 acid (2,4-D)- and 2,4,5-trichlorophenoxyacetic acid (Hayashi et al., 2016). It is noted that prior
268 to our study, no CIP-degrading strain has been isolated from activated sludge. The CIP
269 degrading strain obtained in this study can be a supplementary bacteria source for strategic
270 bioaugmentation of activated sludge treatment.

271 [FIGURE 1]

272 **3.2 CIP biotransformation by the *Bradyrhizobium* sp. strain GLC_01**

273 The time-course removals of CIP over 8 days in four different batch experimental settings that
274 represent removal by biotransformation (I), adsorption (II), utilization of CIP as sole carbon
275 source (III) and abiotic (IV) were presented in Fig. 2a. The observed CIP removal in each
276 experiment were 46.7 ± 1.9% (I), 5.2 ± 1.0 (II), 3.0 ± 1.5 (III) and 2.5 ± 1.0 (IV). The results
277 suggest that the removal pathway of CIP was mainly biotransformation (38 ± 2%). Only a small
278 fraction of CIP was removed by adsorption and abiotic means. The removal of CIP via
279 adsorption and abiotic means depends on its physicochemical properties (e.g. volatility and
280 hydrophobicity). The Henry's constant of CIP is 5.09×10^{-19} atm m^3/mol (Table S1),
281 indicating that CIP has low volatility. The log octanol-water partition coefficient (log K_{ow}) of
282 CIP is 0.28 suggesting that it is hydrophilic and adsorption to activated sludge is insignificant.
283 The results further showed that the biotransformation of CIP occurred via cometabolism.
284 Cometabolism is the transformation of a non-growth substrate in the presence of a growth

285 substrate or another transformable compound. The term 'non-growth substrate' describes
286 compounds that are unable to support cell growth as sole carbon source (Tobajas et al., 2012).
287 Cometabolic transformation of CIP is evident upon comparing experiment I (fed with medium
288 + CIP) and III (fed with CIP only). There was no microbial growth in experiment III, whereas
289 intensive growth occurred in experiment I (OD increases from 0.1 to 1.6). CIP removal at the
290 end of incubation period in experiment III was significantly less ($P < 0.05$ by Student's t-test)
291 than that of experiment I (3.0 ± 1.5% *vs.* 46.7 ± 1.9) (Fig. 2a). CIP removal in experiment I
292 increased sharply after 3 days incubation (from 8.3 ± 0.23%, day 1 to 37.4 ± 2.3, day 3), which
293 coincided with high COD removal and cell growth rate (Fig 2b). Then, CIP removal was stable
294 at 45 ± 2% until the end of incubation period (Fig 2a) after 100% COD was consumed
295 (experiment I). The degradation of CIP followed a first-order kinetics reaction with the reaction
296 rate constant k of 2.53 (1/h) (Fig S1). Taken together, these observations led us to conclude
297 that biological CIP removal occurred via cometabolism rather than direct metabolism by the
298 *Bradyrhizobium* sp. strain GLC_01. Our results suggest that the isolated strain can be a new
299 auxiliary bacterial source for the removal of CIP-containing wastewater.

300 The biotransformation of CIP via cometabolism by activated sludge strain is a notable finding
301 from this study. Previous studies have reported that adsorption and biotransformation were the
302 main removal mechanisms of TrOCs in activated sludge (Luo et al., 2014; Tran et al., 2018).
303 However, biotransformation is preferable to adsorption in activated sludge, because adsorption
304 is not detoxification or mineralization. The adsorbed TrOCs would require further treatment or
305 monitoring in downstream of activated sludge or biosolids (Semblante et al., 2015). The
306 currently study provides ample evidence that an activated sludge strain can perform
307 biotransformation of CIP through cometabolism rather than direct metabolism.
308 Biotransformation of TrOCs in activated sludge likely occurs via cometabolism due to their
309 low concentration to serve as main growth substrate. Moreover, TrOC concentration varies in

310 wastewater. If a compound is not present for an extended period, the specific compound
311 degrader via direct metabolism could be washed out or outcompeted by other species and only
312 the cometabolic organisms can survive (Fischer & Majewsky, 2014). The biotransformation of
313 TrOCs via cometabolism would have important implications for process optimization. For
314 example, prior exposure to CIP is not required due to the cometabolism, but the presence of
315 other substrates is crucial. Thus, altering environmental factors (e.g. primary carbon source
316 concentration and types) can influence the CIP removal. The following section will provide the
317 evaluations on the abiotic factors influencing CIP biotransformation.

318 [FIGURE 2]

319 Biotransformation of CIP by the *Bradyrhizobium* sp. strain GLC_01 was further confirmed by
320 the detection of by-products in the aqueous phase of experiment I (i.e. active cells). The first
321 by-product was proposed as 7-amino-1-cyclopropyl-6-fluoro-4-oxo-1,2,3,4-
322 tetrahydroquinoline-3-carboxylic acid with m/z = 263.2 (Fig S2). This was formed due to the
323 loss of piperazine ring. The results suggest that the N-C bond of the piperazine ring can be
324 biologically break-down by bacteria in this study. In a previous study, N-C bond cleavage by
325 brown-rot fungus has also been reported (Wetzstein et al., 1999). The second by-product with
326 the m/z of 348.1 was proposed as (1-cyclopropyl-6-fluoro-8-hydroxy-4-oxo-7-(piperazinyl-1-
327 y1)-1,2,3,4-tetrahydroquinoline-3-carboxylic acid). This compound was formed by
328 hydroxylation process. The by-products were identified based on mass spectra and the
329 fragmentation patterns and in comparison with the previous studies (Jia et al., 2018; Paul et al.,
330 2010; Wetzstein et al., 1999). This study appears to be the first report the biotransformation of
331 CIP by a microbe originating from activated sludge.

332 **3.3 Abiotic factors optimizing CIP biotransformation**

333 3.3.1 Concentration and type of the primary carbon source

334 Cometabolic transformation of CIP can be influenced by the availability of cometabolites, i.e.
335 the primary carbon source. In this study, the results revealed that COD concentration positively
336 affected CIP biotransformation (Fig. 3a). CIP biotransformation decreased as the concentration
337 of the carbon source (i.e. glucose) decreased. Decreasing the carbon source concentration also
338 led to a decrease in cell growth as indicated by $OD_{620\ nm}$ measurement (Fig. 3b). High COD
339 concentration supported strain GLC_01 growth and consequently influenced the overall CIP
340 biotransformation.

341 Previous studies suggested that initial COD affected the removal of TrOCs via co-metabolic
342 transformation (Pan et al., 2018; Tobajas et al., 2012). For instance, a thermophilic bacteria
343 strain *Thermus sp.* degraded 52% of 5 mg L^{-1} CIP under the addition of 0.5 g L^{-1} sodium
344 acetate and 2 days incubation (Pan et al., 2018). Increasing sodium acetate concentration to 3
345 g L^{-1} inhibited cell growth and CIP degradation. The inhibition of cell growth was possibly
346 caused by high levels of sodium acetate (i.e. more carbon source) that increased the C/N ratio
347 in the culture medium. The C/N ratio in the current study was 57:1 (for the COD of 2000 mg
348 L^{-1}). Although this value was higher than that of a normal biological process (a mixed culture)
349 (25-30:1), no inhibition was observed. Since the organic carbon concentration is the key
350 operating parameter for CIP removal efficiency, strategy to increase the organic carbon
351 concentration should also consider the C/N ratio in future study.

352 [FIGURE 3]

353 The type of primary carbon source has also been reported to affect the removal efficiency of
354 various compounds (Xiong et al., 2017; Zhou et al., 2013). In this study, we examined the
355 effects of different types of readily biodegradable carbon sources sodium acetate, glucose and

356 starch with a diverse range of chemical structures and biodegradable levels. The results showed
357 that type of primary carbon source could affect cell growth and CIP removal. With sodium
358 acetate and glucose in the culture medium, the strain grew at faster rate compared to starch (P
359 < 0.05 by Student's t-test). An exponential phase occurred within 3 days of incubation. The
360 $OD_{620\ nm}$ measurement was much lower in starch medium (0.14 ± 0.01) compared to sodium
361 acetate (1.47 ± 0.03) and glucose (1.62 ± 0.01) (Fig. 4a). Conversely, above 90% of COD was
362 consumed in sodium acetate and glucose medium, whereas only 20% of COD was used in
363 starch ($P < 0.05$ by Student's t-test). The removal efficiency of CIP was 46.7 ± 1.9, 32.5 ± 2.8
364 and 12.5 ± 3.5% (Fig. 4b) in glucose, sodium acetate and starch medium, respectively.

365 [FIGURE 4]

366 Biodegradability of carbon source and physiology of microorganism strains were two main
367 reasons cited for different removal efficiencies by isolates or pure cultures (Fischer &
368 Majewsky, 2014; Zhou et al., 2013). It is likely that the type of primary carbon source
369 influenced the enzymatic system involved in the co-metabolic reaction. Further studies into the
370 metatranscriptomic sequencing of strain GLC_01 under different primary carbon types may
371 reveal the reason for the observation in this study. At this stage, the results provide information
372 for the selection of primary carbon types.

373 3.3.2 Initial concentration of CIP

374 Initial CIP concentrations (0.05 to 10 mg L^{-1}) had no impact on the cell growth. For example,
375 $OD_{620\ nm}$ values of the culture under CIP concentration of 0.05 and 10 mg L^{-1} were similar (1.58
376 *vs* 1.52) ($P > 0.05$ by Student's t-test). Consistently, over 94% of COD was consumed from all
377 the cultures after 3 days of incubations. The results indicated that *Bradyrhizobium* sp. strain
378 GLC_01 could tolerate high levels of CIP. The physiology of the bacterial cell (e.g. cell

379 membrane properties, presence of efflux pump) and the phenotype (e.g. degradation capacity)
380 determine antibiotic resistance of microbes (Oh et al., 2013).

381 CIP concentration influenced the biotransformation capacity of strain GLC_01 (Fig. 5). At the
382 highest CIP concentration (10 mg L^{-1}), the removal efficiency of CIP was 26.4 ± 4.3%. The
383 removal efficiency increased further to 70.4 ± 7.4% when CIP concentration was reduced to
384 0.05 mg L^{-1} ($P < 0.05$ by Student's t-test). The removal efficiency of CIP by *Bradyrhizobium*
385 sp. strain GLC_01 in this study is higher than previous reported values by other organisms (Pan
386 et al., 2018; Xiong et al., 2017). Freshwater microalgae *Chlamydomonas mexicana* degraded
387 only 13% of 2 mg L^{-1} CIP after 11 days of incubation (Xiong et al., 2017). A thermophilic
388 bacteria strain *Thermus thermophilus,* removed 55% of CIP (Pan et al., 2018). The higher
389 degree of CIP removal by *Bradyrhizobium* sp. makes it a potential source of bacteria for
390 bioremediation application.

391 The removal of CIP under the influence of its concentration followed the first-order reaction
392 kinetics. The obtained first-order rate constants are summarized in Table S2. The results
393 showed that the biotransformation rate constants (k) also decreased from 5.03 to 1.05 (h^{-1}) with
394 increased CIP concentration from 0.05 to 10 mg L^{-1}. Overall, the results illustrated that initial
395 CIP concentration negatively correlated with biotransformation rate by the *Bradyrhizobium* sp.
396 strain GLC_01.

397 [FIGURE 5]

398 **4. Conclusion**

399 CIP-degrading *Bradyrhizobium* sp. GLC_01 was isolated from activated sludge. Our
400 quantitative analyses revealed that biotransformation was the major removal pathway of CIP
401 by *Bradyrhizobium* sp. GLC_01, and biotransformation occurred via cometabolism with the
402 presence of another primary carbon source, rather than direct metabolism. Concentration and

403 biodegradability of the primary carbon substrate affected the extent and rate of CIP
404 biotransformation. Higher concentration of the primary carbon substrate led to the higher
405 removal of CIP. The biotransformation of CIP was influenced by the initial CIP concentration.
406 The results of this study provided new insights into devising biological means (e.g.
407 bioaugmentation) of treating CIP-containing wastewater.

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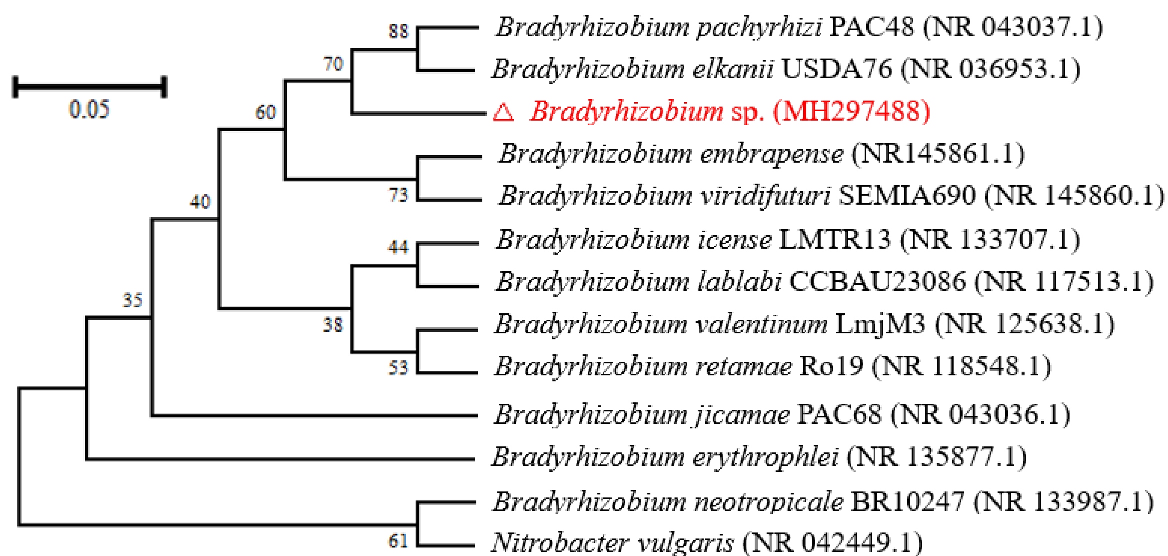
411 **Conflicts of interest**

412 The authors declare that they have no competing interests.

413 **List of Figures**:

414



415

416 **Figure 1**.

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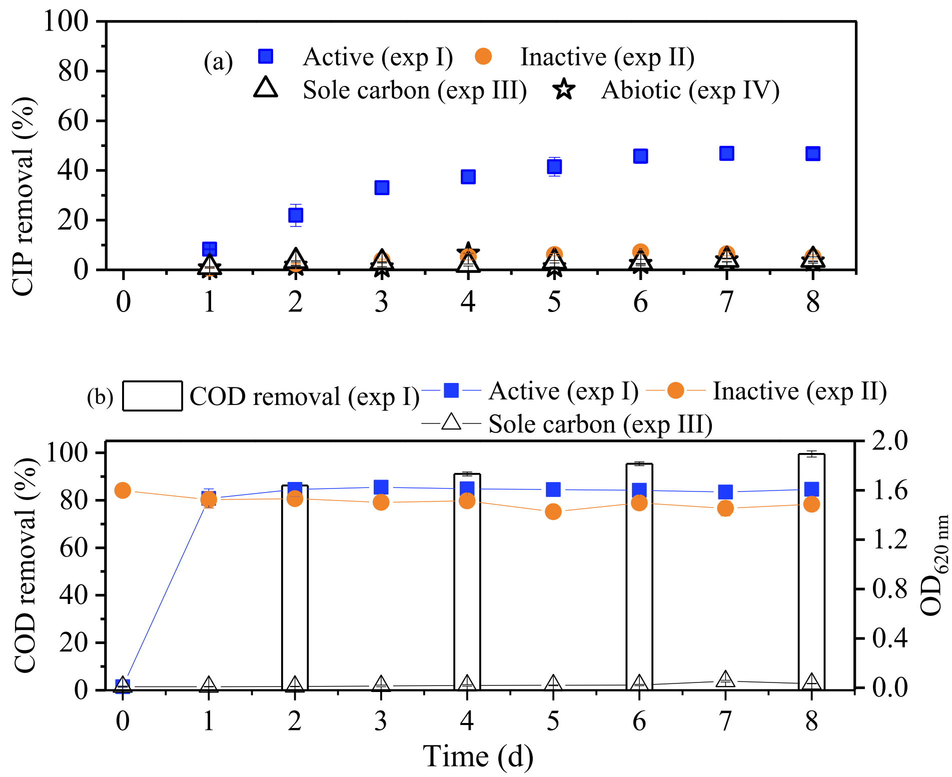

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420 **Figure 2**.

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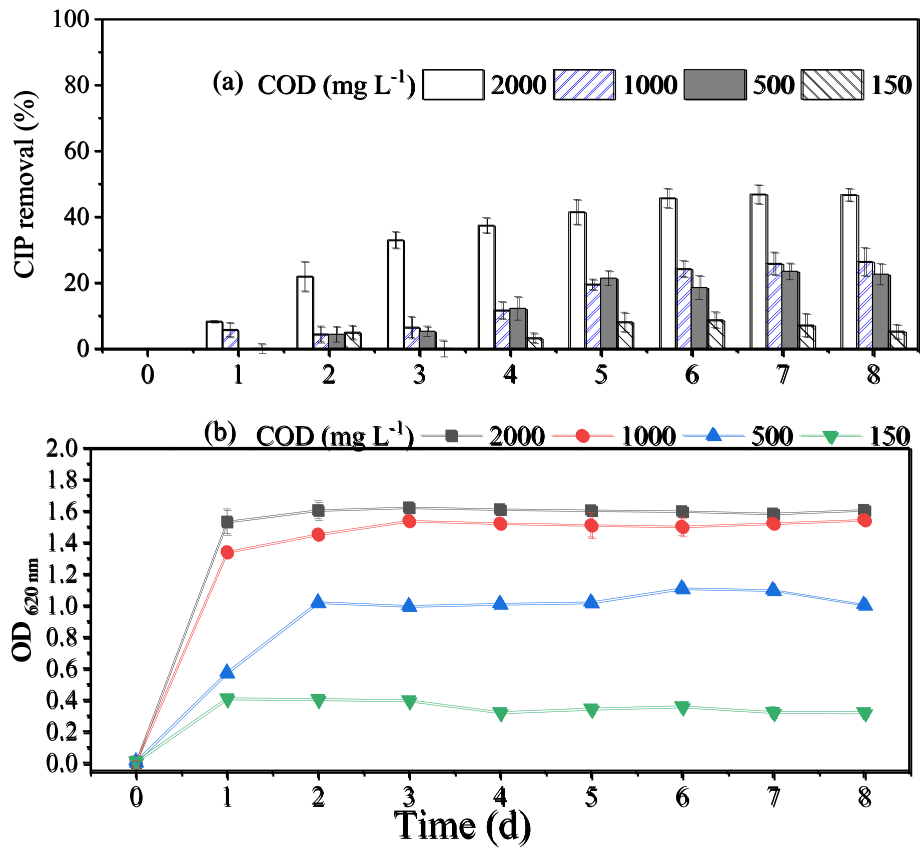

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423 **Figure 3**.

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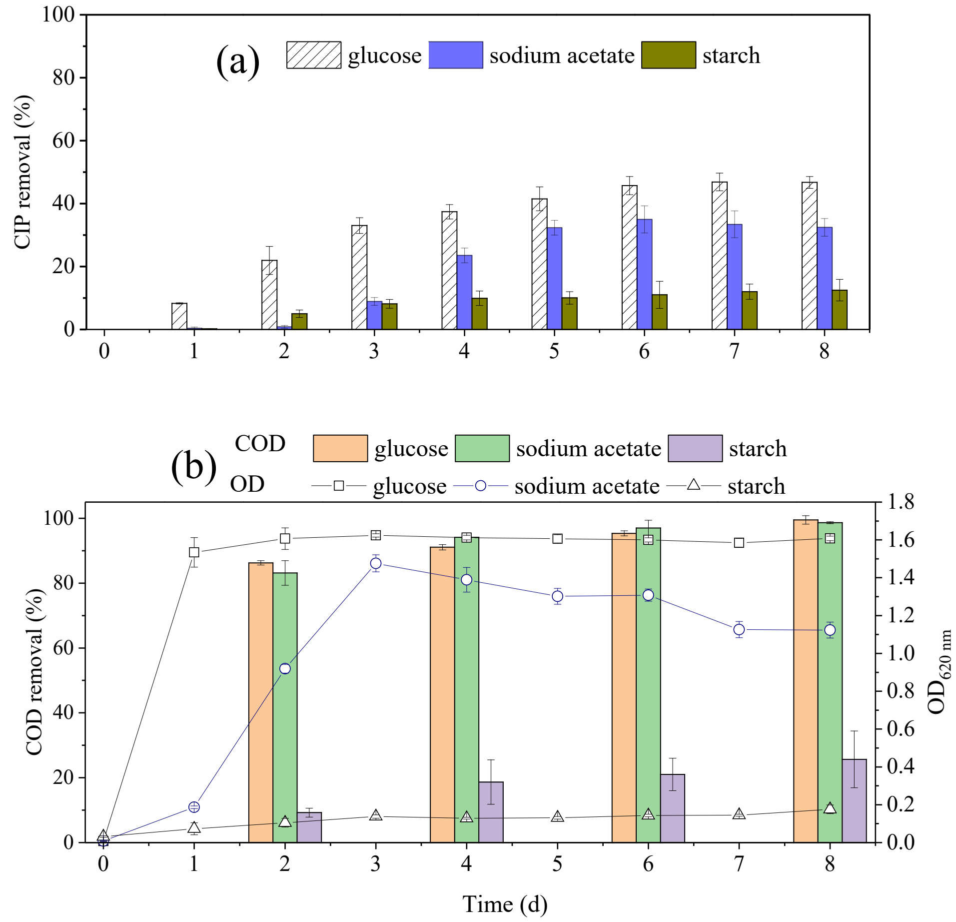

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429 **Figure 4**.

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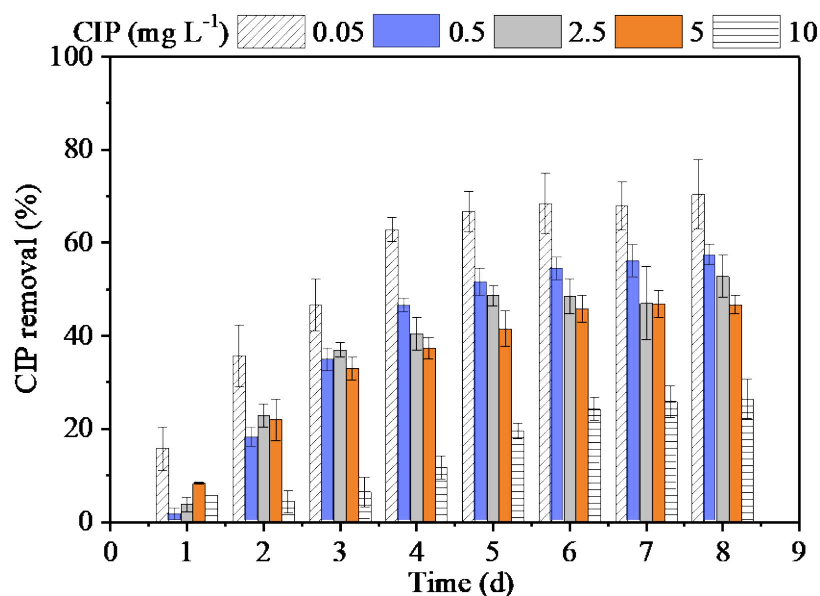

431

432 **Figure 5.**

433

434 **References**

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