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**Microbial Degradation of Pyridine by Co-culture of Two Newly
Isolated Strains, *Arthrobacter* sp. Strain PDC-1 and *Rhodococcus* sp.
Strain HPD-2**

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Running Title: Pyridine degradation by co-cultural strains

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29 **Abstract**

30 Pyridine is one of the most widespread heterocyclic contaminants. Microbial degradation of
31 pyridine seems quite promising for its safety and efficiency. A bacterial consortium, which could
32 use pyridine as the sole source of carbon, nitrogen, was obtained from the petroleum-contaminated
33 soil from Liao River estuarine wetland. Two pyridine degrading strains, designated as PCD-1 and
34 HPD-2, were isolated from the bacterial consortium. PCD-1 was identified as an *Arthrobacter*, and
35 HPD-2 was identified as the *Rhodococcus* genus. The effects of pH, temperature, and pyridine
36 concentration were investigated, and the optimum growth conditions for two strains were similar at
37 pH 7.0 and 30°C. The co-culture of the two strains, CoPD, has better degradation efficiency
38 compared with the individual strain. Haldane's inhibitory growth kinetics equation could be fitted
39 to the growth of co-culture CoPD well for the entire concentration range. The kinetic constants
40 obtained were $\mu_{\max} = 0.141 \text{ h}^{-1}$, $K_s = 37.9 \text{ mg/L}$, and $K_i = 3830 \text{ mg/L}$. Co-culture CoPD was able to
41 remove more than 98% pyridine with an initial pyridine concentration of 5,000 mg/L within 120 h.
42 Strain PCD-1 and HPD-2 have a novel pyridine degradation pathway different from the reported
43 pathways. Major intermediates of pyridine degradation by two strains, including 2,5-pyrroldione,
44 maleic semialdehyde, furanone, and butyrolactone, were identified using LC-MS analysis. CoPD is
45 a promising tool for the treatment of wastewater containing pyridine, and this study contributes to
46 the knowledge of the pyridine biodegradation by bacterial consortium.

47

48 **Introduction**

49 Pyridine and its derivatives are of major concern as carcinogenic, teratogenic, and mutagenic
50 environmental pollutants. Pyridine is classified as a hazardous substance in the USEPA list of

51 priority pollutants (Richards and Shieh, 1986), and is extensively used in industries as solvent and
52 platform chemical for the synthesis of textiles, pharmaceuticals, agrochemicals and industrial
53 manufacturing (Scriven and Murugan, 1996; Sims et al., 1989). Pyridine is volatile, the
54 hetero-aromatic ring makes it persistent in the environment, and the nitrogen atom gives the
55 hydrophilic properties to pyridine, which makes it better dissolved in water than its carbocyclic
56 analogs. The pyridine and its oxidized derivatives may cause severe health implications (Sun et al.,
57 2013). Therefore, removing pyridine from the environment or wastewater has long caught the
58 researchers' attention (Bouyarmane et al., 2010; Li et al., 2017; Sims et al., 1989). The existing
59 physicochemical purification methods are not very efficient when they treat nitrogen heterocyclics
60 (Chu et al., 2018; Li et al., 2017). Therefore, the biological degradation of pyridine is considered to
61 be an attractive approach compared with physical or chemical methods. Pyridine degradation by
62 strain(s) individually or immobilized strain(s) on fixing agents in bioreactors is a subject of a good
63 deal of research (Lin et al., 2010; Uma and Sandhya, 1998; Wen et al., 2013). In general, the
64 pyridine strain with high pyridine degrading capacity will enhance the pyridine degradation
65 capacity in the bioreactor through bioaugmentation (Jin et al., 2020; Liu et al., 2020; Lodha et al.,
66 2008; Uma and Sandhya, 1998; Wen et al., 2013; Xue et al., 2020; Zhang et al., 2018). Therefore,
67 the study of pyridine degrading strain(s) is one of the core issues of pyridine removal. To data,
68 several strains have been isolated from the environment which could use pyridine as the sole
69 source of carbon and nitrogen, including *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Azparcus*,
70 *Bacillus*, *Gprdpmoa*, *Micrococcus*, *Nocardioides*, *Nocardia*, *Paracoccus*, *Pseudomonas*,
71 *Rhizobium*, *Rhodococcus*, *Rhodopseudomonas*, *Shewanella*, *Shinella* et al (Jin et al., 2020; Liu et
72 al., 2020; Mathur et al., 2008; Wang et al., 2018; Xue et al., 2020; Zhang et al., 2018). The isolates

73 presented different degradation capacities. Some strains could grow with 5500 mg/L initial
74 concentration of pyridine and completely removed the pyridine within 192 hours (Stobdan et al.,
75 2008), while some strain could bear only 20 mg/L pyridine and the degradation efficiency was
76 dropped significantly with 30-50 mg/L pyridine (Kaiser and Bollag, 1992; Lodha et al., 2008).
77 Therefore, the high pyridine tolerance and efficient pyridine degradation strain still to be
78 elucidated.

79 In the wild, the pyridine degrading strain lives in a complex environment with other nutrients or
80 other strains. All the elements will influence the pyridine degradation (Chandra et al., 2009). The
81 pyridine degradation capability will be enhanced by adding some nutrients such as glucose as well
82 as other pollutants such as phenol (Bai et al., 2009; Quan et al., 2017; Sun et al., 2011). Besides,
83 other elements such as the products of pyridine degradation, ammonia, will also affect the pyridine
84 degradation (Qiao and Wang, 2010; Wang et al., 2018). Pyridine degradation will also be
85 influenced by fixation methods, as well as bioreactor and zeolite (Bai et al., 2010; Wen et al., 2013).
86 Co-cultures of bacteria for pyridine degradation has also been studied. In general, mixed culture
87 was usually more effective for pyridine degradation compared with individual strain, which might
88 due to the complementary metabolism of different strains (Chandra et al., 2010). However, the
89 function of different strains in pyridine degradation was not fully understood. The research of the
90 co-cultures for pyridine degradation will help us to elucidate the interaction between strains and
91 the adaptation to the environment.

92 In this study, a pyridine-degrading bacterial consortium was obtained and two single strains with
93 pyridine degrading capacity were isolated from the bacterial consortium. The degradation
94 properties of the co-culture of the two isolates were investigated. The growth kinetics was

95 investigated for better understanding the pyridine degradation. In addition, the metabolic products
96 of the two strains were also analyzed.

97

98 **Materials and methods**

99 **Chemicals**

100 Pyridine, 2-hydroxypyridine, 3-hydroxypyridine, 4-hydroxypyridine and monocarboxylic
101 pyridines were of analytical grade and purchased from Aladdin (Shanghai, China). Methanol and
102 formic acid were LC-MS grade and purchased from Aladdin (Shanghai, China) and ThermoFisher,
103 respectively. All other chemicals used in this study were of AR grade with more than 99% purity
104 and purchased from commercial manufacturers.

105 **Strains and cultivation conditions**

106 Pyridine-degrading bacterial consortium was isolated from petroleum-contaminated soil taken near
107 the Liao River estuarine wetland. Single colonies were obtained by plating the bacterial consortium
108 on the MSM agar plate with particular substrates as previously described (Yu et al., 2018). The
109 bacterial consortium/strains were grown in 250 mL Erlenmeyer flasks containing 50 mL MSM
110 with different concentrations of pyridine unless otherwise stated. MSM was sterilized by
111 autoclaving at 121°C for 20 min. Pyridine or other pyridinic compounds were added after filtered
112 by a 0.22 µm sterile syringe filter (Jinteng, China). The strains were incubated at 30°C on a rotary
113 shaker (150 rpm). Isolated strains were identified by 16S rRNA gene sequence analysis. The
114 genomes of bacteria were extracted using Ezup pillar bacterial genome DNA extraction kit
115 (Sangon Biotech, China) followed the instruction for Gram-positive strains. The 16S rRNA gene
116 was amplified using primers 27F and 1492R. The PCR reaction was performed using the following

117 cycling conditions: 95°C for 5 min, 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 90 s,
118 followed by 72°C for 10 min. Nucleotide sequences similarities for phylogenetic analysis were
119 obtained from the LPSN database. Phylogenetic analysis was performed by MEGA6.0 software.

120 **Biodegradation of pyridine by Co-culture or isolated strains**

121 In order to understand the degradation and growth pattern of bacterial co-culture or isolates, the
122 effects of pH, temperature, and substrate concentration were determined in MSM. The samples
123 were withdrawn at particular time interval and analyzed for bacterial growth. The cells were then
124 removed by centrifugation at $10,000 \times g$, 4°C for 5 min, and the supernatant was transferred to a
125 new centrifugation tube for further analysis. Control experiments were carried out using
126 autoclaved MSM with the same pyridine concentration without microorganisms to determine the
127 loss of substrate due to evaporation.

$$\text{Degradation rate} = \left(1 - \frac{\text{pyridine concentration in the sample}}{\text{pyridine concentration in the control}}\right) \times 100$$

128 **Kinetics models**

129 In order to understand the growth pattern of the co-culture. The effects of substrate concentration
130 were performed in MSM with different concentrations of pyridine. In the batch system, the specific
131 growth rate (μ) was defined as

$$132 \quad \frac{dX}{dt} = \mu X - K_d X \quad (1)$$

133 X is cell concentration (OD600nm), μ is a specific growth rate of the cell (h^{-1}), and K_d is the
134 endogenous decay coefficient (h^{-1}). At the exponential growth phase, the K_d could be neglected,
135 and the equation (1) was reduced to

$$136 \quad \frac{dX}{dt} = \mu X \quad (2)$$

137 The value of μ was determined at the exponential phase of the growth curve according to equation

138 (2).

139 Two models were used to fit the experimental data obtained from the batch experiments. Monod
140 model represents bacterial growth under substrate-limited and non-inhibitory conditions as
141 equation (3).

$$142 \quad \mu = \frac{\mu_{max}S}{K_s+S} \quad (3)$$

143 Haldane inhibitory growth model represents the growth kinetics of an inhibitory compound such as
144 pollutants (Kumar et al., 2005; Park et al., 2002) as equation (4).

$$145 \quad \mu = \frac{\mu_{app}*S_0}{K_s+S_0+(\frac{S_0^2}{K_i})} \quad (4)$$

146 where S_0 denotes the concentration of pyridine (mg/L).

147 The true maximum specific growth rate in the Haldane model occurred when $d\mu/dS=0$ as below
148 (Christen et al., 2012):

$$149 \quad S_m = \sqrt{K_s K_i} \quad (5)$$

150 The maximum specific growth rate can be obtained by replacing S_m in Eq. (4).

151 The values of the kinetic parameters were obtained from non-linear fitting using Origin software.

152 **Intermediates identification and phylogenetic analysis**

153 Resting cell suspensions were prepared as previously described (Yu et al., 2018). The bacteria in
154 the early stationary phase were collected by centrifugation at 8,000×g, 4°C for 5 min, washed
155 twice with phosphate salt buffer, and resuspended with double distilled water. The resting cell
156 reactions were performed in 250 mL flask at 30°C 150 rpm. The samples were collected at interval
157 times. The cells were removed by centrifugation at 10,000 × g for 2 min. The supernatant was
158 transferred to a new tube and stored at -20°C for further analysis.

159 **Analytical methods**

160 Growth was monitored spectrophotometrically by measuring the absorbance at wavelength 600 nm
161 (OD₆₀₀). Pyridine concentration was monitored by high-performance liquid chromatography
162 (HPLC) (Waters Alliance HPLC, USA) equipped with a column of Waters Spherisorb ODS2 (4.6
163 mm × 250 mm) and a PAD detector. The mobile phase was 70% (v/v) methanol and 30% 0.05%
164 (w/v) formic acid at a flow rate of 1 mL/min. The signal of 265 nm was monitored to calculate the
165 pyridine concentration. The supernatant of the samples was treated by adding two-volume of
166 methanol. The samples were precipitated at 4°C for 5 min, centrifuged at 10,000 × g for 2 min, and
167 then filtered by a 0.22 µm sterile syringe filter.

168 To identify the degradation intermediates of the two isolated strains, samples from resting cell
169 reactions were analyzed by LC-MS/MS. LC-MS/MS analysis was performed with UltiMate3000
170 UHPLC system (Thermo Fisher, USA) equipped with Orbitrap Fusion Lumos mass spectrometer
171 and an electrospray ionization source (ESI), using reverse-phase column (Agilent ZORBAX
172 RRHD Eclipse Plus 95Å C18 (2.1 × 100 mm, 1.8 µm) at 30°C. The mobile phase is 70% (v/v)
173 methanol and 30% 0.05% (w/v) formic acid at a flow rate of 0.2 mL/min. Both positive and
174 negative electrospray ionization analysis with the continuous full scanning from *m/z* 50 to 500
175 were performed. The data were analyzed using Compound Discovery software (Thermo Fisher).

176

177 **Results**

178 **Strains identification**

179 A bacterial consortium, which could utilize pyridine as the sole source of carbon and nitrogen, was
180 isolated from petroleum-contaminated soil in Liao River estuarine wetland. A series of pyridinic

181 compounds were provided as growth substrates for this bacterial consortium, respectively. The
182 bacterial consortium could grow with pyridine and 2-hydroxypyridine, but it could not use
183 3-hydroxypyridine, 4-hydroxypyridine, picolinic acid, nicotinic acid, or isonicotinic acid as the
184 growth substrate. After serial dilution, one dominant colony was observed on the MSM + pyridine
185 agar plate. This strain was isolated and the 16S rRNA gene was sequenced. This strain was
186 designated as PCD-1 and classified as *Arthrobacter* based on the 16S rRNA gene and morphology
187 properties. However, PCD-1 could only grow with pyridine, it could not grow with
188 2-hydroxypyridine. Then, this bacterial consortium was cultivated with 2-hydroxypyridine as the
189 sole substrate for 3 cycles. After serial dilution, one dominant colony was isolated from the plate.
190 This strain, namely HPD-2, was identified as *Rhodococcus* sp. Strain HPD-2 could grow with both
191 pyridine and 2-hydroxypyridine as the substrate.

192 **Effect of temperature and pH on the degradation of pyridine**

193 The growth and pyridine degradation were assessed at different pH and temperature values for
194 both *Arthrobacter* sp. PCD-1 and *Rhodococcus* sp. HPD-2. The results revealed that strain PCD-1
195 could grow well at a temperature ranging from 25°C to 37°C, and showed the highest growth rate
196 at 30°C (Fig. 1a). Strain PCD-1 grew well at pH values ranging from 7.0 to 9.0 and showed the
197 highest growth rate at pH 8.0 (Fig. 1b). However, there was almost no growth at pH 6.0 or pH 10.0
198 for strain PCD-1. The optimum pH values were observed to be 7.0-9.0 for *Rhodococcus* sp. HPD-2.
199 The lag phase at pH 7.0 was slightly shorter than those at pH 8.0 and 9.0, and the maximum cell
200 density at pH 9.0 was a little bit higher than those at pH 7.0 and 8.0 (Fig. 1c). The optimum
201 temperature for strain HPD-2 was 30°C (Fig. 1d). The results revealed that both strains could grow
202 well with pyridine as the substrate, and the optimum growth conditions for the two strains were

203 very similar. *Rhodococcus* sp. strain HPD-2 could use both pyridine and 2-hydroxypyridine as the
204 growth substrate. 2-Hydroxypyridine is a better growth substrate for strain HPD-2 than pyridine
205 (Fig. 2a). The culture color of strain HPD-2 with pyridine was white, while the culture color with
206 2-hydroxypyridine was yellow-brown (Fig. 2b).

207 **Growth kinetics of co-culture**

208 Both *Arthrobacter* sp. PCD-1 and *Rhodococcus* sp. HPD-2 could use pyridine as the growth
209 substrate, thus the two strains were re-mixed to form the co-culture CoPD. The growth with
210 co-culture and individual strain were compared. It was observed that the growth of individual
211 strain *Arthrobacter* sp. PCD-1 and *Rhodococcus* sp. HPD-2 was slower compared to the co-culture
212 of the two strains (Fig. 3). Under optimal conditions, the growth of co-culture, CoPD, was
213 measured with different concentrations (from 100 mg/L to 5000 mg/L) of pyridine. The co-culture
214 could grow in all the tested concentrations. The lag phase with high initial pyridine concentration
215 (≥ 2000 mg/L) was significantly prolonged, which may be due to substrate inhibition (Fig. 4a).
216 Figure 4 shows a typical trend that the value of specific growth rate increases with the increase of
217 initial pyridine concentration up to a certain concentration level, then it begins to decrease with the
218 increase of pyridine concentration. The results confirmed that pyridine was an inhibitory substrate.
219 The growth kinetics are essential for understanding the capacities of the bacteria for pollutant
220 degradation. Therefore, the determination of the growth kinetics of microorganisms has been one
221 of the main issues in these studies. Two growth models were used to describe the growth rate of
222 co-culture CoPD for a wide pyridine concentration. In general, the Haldane's growth model is used
223 to describe the growth kinetics data of an inhibitory compound. The correlation coefficients (R^2)
224 also showed that the Haldane inhibition model is the better kinetic model for the co-culture CoPD

225 growth with pyridine(Fig. 4b). The value of maximum specific growth rate, $\mu_{\max}$, is 0.141 h⁻¹ at 380
226 mg/L (S_m) initial pyridine concentration, and the substrate inhibition coefficient, K_i , is 3830 mg/L.
227 The value of the substrate half-saturation coefficient, K_s , is 37.9 mg/L.

228 The effects of initial concentration on the degradation behavior of pyridine were conducted. The
229 initial concentration of pyridine was varied from 100 to 5000 mg/L. The degradation data are given
230 in Figure 4c, d. Co-culture CoPD could completely degrade all the tested pyridine concentrations
231 ranging from 100 to 5000 mg/L to an efficiency of >98%.

232 **Degradation profile of pyridine by two strains**

233 To investigate the degradation pattern of pyridine by two strains, resting cell reactions were
234 performed with different substrates including pyridine, 2/3/4-hydroxypyridine or
235 2/3/4-carboxypyridine. The samples were analyzed using HPLC, and the results showed that strain
236 PCD-1 could only convert pyridine, while strain HPD-2 could only convert pyridine and
237 2-hydroxypyridine. A single peak of intact pyridine compound was observed at a retention time of
238 3.56 min in the HPLC chromatogram, and the peak disappeared gradually with the reaction time.
239 However, no new peak could be detected in the HPLC signal.

240 The intermediates were then identified using high-resolution accurate mass measurements by
241 Orbitrap Fusion Lumos (Thermo Scientific) (Fig. 5). Two main intermediates detected in the
242 resting cell reaction of strain PCD-1 were presented. The base peak of M1 with m/z of 98.06020 in
243 the positive ion ESI mass spectrum in Figure 5a was identified as 2,5-pyrrolidione, which matched
244 with formula C₄H₃NO₂. Compounds M2 and M3 which were detected with an m/z of 87.08062 and
245 101.09622, respectively, were identified as butyrolactone (C₄H₆NO₂) and maleic semialdehyde
246 (C₄H₄NO₃), each. For strain HPD-2, the intermediate M2 was also detected in the LC-MS spectra

as shown in Figure 5c. Besides, a peak of M4 with an m/z of 85.02858 in the positive ion ESI mass spectrum was identified as furanone ($C_4H_4O_2$) as shown in Figure 5d. It can be seen that M2 could be produced by both strain PCD-1 and HPD-2. Besides, M3 and M4 are the substrate and product of a catalyzing reaction. Thus, it can be proposed that the two strains use similar pyridine degradation pathway, which was proposed as Figure 6 based on the intermediates' identification and previous studies.

Discussions

Environmental pollution has become one of the serious problems in modern society. Millions of tons of pollutants are discharged every day, among which pyridine and its derivatives are one of the most distributed contaminants. Microbial bioremediation is a promising method for pyridine removal from the environment. In this study, two newly isolated strains, *Arthrobacter* sp. PCD-1 and *Rhodococcus* sp. HPD-2, which could use pyridine as the sole source of carbon and nitrogen, were isolated from the petroleum pollutant soil in Liao River estuarine wetland. The two isolates were mixed to form bacterial consortium CoPD. Both *Arthrobacter* and *Rhodococcus* were widely studied because of their properties of degradation for recalcitrant aromatic and pyridinic compounds (Arora and Sharma, 2015; Presentato et al., 2018; Ren et al., 2018; Sengupta et al., 2019; Sun et al., 2011; Yao et al., 2012; Zefirov et al., 1994). Compared with reported *Arthrobacter* and *Rhodococcus*, the co-culture CoPD showed more excellent pyridine degradation capacity. Besides, CoPD showed better pyridine biodegradation capacity than each of the two isolated strains. CoPD was able to completely degrade pyridine at an initial concentration up to 5,000 mg/L. This is a high reported pyridine concentration among all the reported pyridine degrading bacteria, and only a few strains have been reported grown with such high pyridine concentration. The

270 calcium alginate gel beads immobilized *Pimelobacter* sp. could remove nearly 90% pyridine at an
271 initial concentration of 10000 mg/L within 164 h (Lee et al., 1994). *Gordonia terre*a IIPN1 could
272 completely remove 5530 mg/L pyridine within 192 days (Stobdan et al., 2008). *Achromobacter* sp.
273 DN-06 could grow with up to 4,300 mg/L pyridine and the $\mu_{\max}$ fitting to Haldane's inhibitory
274 growth kinetics was 0.161 h^{-1} , similar to that of co-culture CoPD (Deng et al., 2011). The excellent
275 pyridine capacity of CoPD might due to the efficient pyridine catalyzing enzymes and the high
276 pyridine tolerance. The growth kinetics studies have proved that high pyridine concentration will
277 inhibit the growth of co-culture CoPD. It has been observed that the lag phase in high pyridine
278 concentration is long. This was probably due to the RNA and protein involved in pyridine
279 degradation that should be synthesis in this phase, therefore high inoculation will reduce the lag
280 phase (Lee et al., 1994). The toxic and inhibitory effect toward the bacterial cells is another reason
281 for the long lag phase. Pyridine is volatile compounds, and the concentration will decrease during
282 the cultivation in both of the control and inoculated culture, especially with high pyridine
283 concentration. At high concentrations, the decrease of pyridine concentration in early-stage mainly
284 due to evaporation. The growth of the strain after a long lag phase may be due to the pyridine
285 concentration has decreased to a relatively low concentration by volatilization. Nevertheless, the
286 co-culture CoPD was able to tolerate and grow on pyridine up to 5000 mg/L.

287 It is important to analyze the degradation pathway of the pyridine, because microbial
288 degradation may produce some carcinogenic intermediates. Several different pathways have been
289 investigated for microbial degradation of pyridine (Khasaeva et al., 2011; Shukla, 1984; Shukla
290 and Kaul, 1986; Wang et al., 2018; Zefirov et al., 1994). The reported microbial degradation
291 pathways could be classified into two different strategies. One strategy is hydroxylation strategy, in

292 which a hydroxylate pyridinic intermediate, such as 2-hydroxypyridine, 2,5-dihydroxypyridine,
293 2,3-dihydroxypyridine, or 2,3,6-trihydroxypyridine, was produced (Fig. 6a). In this strategy, at
294 least two hydroxyl groups were added into the pyridine-ring, which subsequently undergo ring
295 cleavage catalyzed by ring-cleavage dioxygenases. The hydroxylate pyridine will polymerize or
296 oxidize to form pigment intermediates, therefore, this strategy can be easily identified by the
297 culture color. The other strategy is dehydrogenation strategy, in which the pyridine ring is
298 hydroxylated to form pyridinic quinones, such as 1,4-dihydroxypyridine,
299 2,3-dihydro-pyridine-2,3/2,4-diol, or piperidine-2-ol, and the pyridinic quinone intermediates are
300 usually unstable, which will automatically form ring cleavage product (Fig. 6b). Thus, in most
301 cases, the cultures of strain have no color and the intermediates could not be detected by the HPLC
302 analysis with an ultraviolet detector.

303 Four degradation intermediates were identified in the present study, including 2,5-pyrrolidione,
304 butyrolactone, maleic semialdehyde, and furanone. Butyrolactone and furanone were only detected
305 in the pyridine degrading strain using hydroxylation strategy (Fig. 6). *N*-formylmaleamic acid was
306 the upstream substrate of butyrolactone and furanone (Fig. 6) and it was produced through
307 degradation of 2,5-dihydroxypyridine or 2,3,6-trihydroxypyridine (Khasaeva et al., 2011; Yu et al.,
308 2015). As was reported by other researchers, 2,3,6-trihydroxypyridine, also called nicotine blue, is
309 a blue pigment, therefore, the culture will turn to blue or brown with a trace amount of
310 2,3,6-trihydroxypyridine (Yao et al., 2012). Besides, 2,5-dihydroxypyridine will also bring blue
311 and brown color for the culture (Tang et al., 2013; Yu et al., 2015). However, the cultures of PCD-1,
312 HPD-2 and CoPD in MSM+pyridine were all white. No new peak could be observed in resting cell
313 reactions by HPLC analysis, which indicated that no hydroxylated pyridine intermediate was

accumulated. Moreover, the co-culture CoPD could not grow with pyridine *N*-oxide. The results indicated that strain PCD-1 and HPD-2 use dehydrogenation strategy to degrade pyridine. Combined with the analysis result of Compound Discovery software, we proposed that the two strains utilize a different degradation pathway from the reported strains. The pyridine degradation was supposed to be initiated via a oxidation reaction to form 2,3-dihydro-pyridine-2,3-diol. 2,3-Dihydro-pyridine-2,3-diol was transformed to *N*-formylmaleamic acid, which was subsequently transformed to 2,5-pyrroldione, maleic semialdehyde, furanone, and butyrolactone. The butyrolactone was converted to form succinic semialdehyde and succinic acid, which enters the TCA cycle.

The better pyridine degradation capacity for CoPD compared with individuals indicated that there are interactions between the two strains in CoPD. The two strains have similar adaptive capacity to the environment and the same degradation pathway. Therefore, they could exchange and use each other's intermediates freely. The higher efficiency might be due to that rate-limiting steps of the two strains are different, therefore, they could share the accumulated metabolic intermediates with each other during pyridine degradation to break the rate-limiting steps. The high pyridine degradation capacity indicated that the two strain has efficient catalyzing enzymes and a special pyridine tolerant system. Therefore, further study will focus on the molecular mechanism of pyridine degradation and tolerance.

Declaration

Ethics approval and consent to participate: This article does not contain any studies with human participants or animals performed by any of the authors.

336 **Consent for publication:** We are very pleasure to agree the paper to be published in AMB
337 Express. The Author grants the Publisher the sole and exclusive license of the full copyright in the
338 Contribution, which license the Publisher hereby accepts. The Author declares that any person
339 named as co-author of the contribution is aware of the fact and has agreed to being so named.

340 **Availability of data and material:** The nucleotide sequence data for strain PCD-1 and HPD-2
341 have been submitted to the GenBank and EMBL nucleotide sequence databases under accession
342 number MK053924 and MK208707, respectively.

343 **Competing interests:** The authors declare that the research was conducted in the absence of any
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349 analyzed the data. XC, LG and HY provided materials, conceived the project and wrote the
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352

353

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459 **Figure Legends**

460 **Figure 1. The temperature and pH influence on PCD-1 and HPD-2 growth were performed**
461 **in MSM with 1000 mg/L pyridine.** (a) Effects of temperature for growth of strain PCD-1; (b)
462 Effects of pH for growth of strain PCD-1; (c) Effects of temperature for growth of strain HPD-2; (d)
463 Effects of pH for growth of strain HPD-2. Each value is the mean from three parallel replicates $\pm$
464 SD.

465 **Figure 2 Growth of *Rhodococcus* sp. strain HPD-2 with 1000 mg/L pyridine and 1000 mg/L**
466 **2-hydroxypyridine, respectively.** (a) Growth curve of strain HPD-2 with different substrates. (b)
467 Cultures of strain HPD-2 with two different substrates. Each value is the mean from three parallel
468 replicates $\pm$ SD.

469 **Figure 3 Growth of co-culture CoPD and the individual two strains in MSM with 1000 mg/L**
470 **pyridine.** Each value is the mean from three parallel replicates $\pm$ SD.

471 **Figure 4 Growth kinetics of co-culture.** (a) Effects of pyridine concentration on the growth of
472 co-culture of strain PCD-1 and HPD-2; (b) Haldane's inhibition growth model fitted to results of
473 batch growth experimental data to determine bio-kinetics parameters. (c, d) Effects of initial
474 pyridine concentration on pyridine degradation (c) and degradation rate (d) of co-culture. Each
475 value is the mean from three parallel replicates $\pm$ SD.

476 **Figure 5 LC-MS analysis of pyridine degradation intermediates of *Arthrobacter* sp. PCD-1 (a,**
477 **b) and *Rhodococcus* sp. HPD-2 (c, d).**

478 **Figure 6 Proposed microbial pyridine degradation pathways.** (a) The hydroxylation strategy of
479 pyridine degradation; (b) The dehydrogenation strategy of pyridine degradation. The red structural
480 formulas indicated the proposed degradation pathway in strain PCD-1 and strain HPD-2.

a

b

491 **Figure 3**

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495 **Figure 4**

Figures

Figure 1

The temperature and pH influence on PCD-1 and HPD-2 growth were performed in MSM with 1000 mg/L pyridine. (a) Effects of temperature for growth of strain PCD-1; (b) Effects of pH for growth of strain PCD-1; (c) Effects of temperature for growth of strain HPD-2; (d) Effects of pH for growth of strain HPD-2. Each value is the mean from three parallel replicates $\pm$ SD.

Figure 2

Growth of *Rhodococcus* sp. strain HPD-2 with 1000 mg/L pyridine and 1000 mg/L 2-hydroxypyridine, respectively. (a) Growth curve of strain HPD-2 with different substrates. (b) Cultures of strain HPD-2 with two different substrates. Each value is the mean from three parallel replicates $\pm$ SD.

Figure 3

Growth of co-culture CoPD and the individual two strains in MSM with 1000 mg/L pyridine. Each value is the mean from three parallel replicates $\pm$ SD

Figure 4

Growth kinetics of co-culture. (a) Effects of pyridine concentration on the growth of co-culture of strain PCD-1 and HPD-2; (b) Haldane's inhibition growth model fitted to results of batch growth experimental data to determine bio-kinetics parameters. (c, d) Effects of initial pyridine concentration on pyridine degradation (c) and degradation rate (d) of co-culture. Each value is the mean from three parallel replicates $\pm$ SD.

Figure 5

LC-MS analysis of pyridine degradation intermediates of *Arthrobacter* sp. PCD-1 (a, b) and *Rhodococcus* sp. HPD-2 (c, d).