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1 **Enhanced dark hydrogen fermentation of *Enterobacter aerogenes*/HoxEFUYH**

2 **with carbon cloth**

3

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22

23 **Abstract**

24 Long-range extracellular electron transfer through microbial nanowires is critical
25 for efficient bacterial behaviors. The application of carbon cloth on the dark hydrogen
26 fermentation using transgenic *Enterobacter aerogenes* (*E. aerogenes/HoxEFUYH*)
27 was first proposed to enhance hydrogen production from glucose. Scanning electron
28 microscopy images showed that the microbial nanowires between *E.*
29 *aerogenes/HoxEFUYH* cells almost vanished due to the presence of carbon cloth.
30 Approximately 59.1% of microorganisms concentrated in biofilms on the surface of
31 carbon cloth, which probably promoted the intercellular electron transfer. The results
32 from Fourier transform infrared spectra and Excitation Emission Matrix spectra
33 indicated that carbon cloth biofilms primarily included polysaccharide and protein.
34 Moreover, the fluorophore of biofilms (88.1%) was much higher than that of
35 supernatant (11.9%). The analysis of soluble metabolic degradation byproducts
36 revealed that carbon cloth selectively enhanced the acetate pathway
37 ($C_6H_{12}O_6+2H_2O\rightarrow 2CH_3COOH+2CO_2+4H_2$), but weakened the ethanol pathway
38 ($C_6H_{12}O_6\rightarrow 2C_2H_5OH+2CO_2$). With 1.0 g/L carbon cloth, the hydrogen yield
39 increased by 26.6% to 242 mL/g, and the corresponding peak hydrogen production
40 rate increased by 60.3%.

41 **Keywords:** Electro-conductive carbon cloth; Transgenic *Enterobacter aerogenes*;
42 Hydrogen fermentation

43

44 1. Introduction

45 Alternative renewable energy has become increasingly important because of the
46 rapid depletion of non-renewable fossil fuels (e.g., coal, petroleum and natural gas)
47 [1]. Hydrogen is a clean carbon-free fuel that plays an important role in reducing
48 greenhouse gas emissions [2]. Currently hydrogen is mainly produced from fossil
49 fuels and water electrolysis [3], which are usually unsustainable, consuming energy
50 and emitting greenhouse gas [4]. Biohydrogen production by dark fermentation offers
51 the advantages of energy-saving, low operating costs, and favorable carbon balances
52 [3, 5]. Facultative anaerobes, such as *Enterobacter*, and strict anaerobes, such as
53 *Clostridium*, are efficient hydrogen producers among a large number of
54 hydrogen-producing microorganisms. *Enterobacter aerogenes* (*E. aerogenes*) shows
55 promising use for dark fermentation due to its high growth and hydrogen production
56 rates [6]. Hydrogen is produced by *E. aerogenes* through the following two pathways
57 [7]: formate decomposition pathway, which evolves hydrogen through formate
58 hydrogenlyase from the formate produced by glycolysis; and nicotinamide adenine
59 dinucleotide (NADH) pathway, which produces hydrogen by hydrogenase through the
60 re-oxidation of NADH produced via glycolysis. Hydrogen production from *E.*
61 *aerogenes* can be regulated by adding external NADH and NAD⁺ [8]. Hydrogenase
62 activity can be enhanced via genetic methods to improve hydrogen production. The
63 hydrogenase genes (*hoxEFUYH*) of *Cyanobacteria Synechocystis* sp. PCC 6803 have
64 been successfully amplified and heterologously expressed in *E. aerogenes*

65 ATCC13408 to improve hydrogen production [9].

66 Methods focusing on parameter optimization (e.g., pH, temperature and substrate
67 concentration) and metabolic bioengineering have been extensively investigated to
68 improve the hydrogen yield and production rate of *Enterobacter* strains [6, 10].
69 However, the hydrogen yield of *E. aerogenes* remains markedly lower than the
70 theoretical hydrogen yield of 4 mol/mol glucose. Various electro-conductive and
71 carbon-based materials, such as metal nanoparticles (NPs) [11-15], biochar [16], and
72 granular activated carbon (GAC) [17], have been recently used to enhance
73 fermentative hydrogen production. Nasy et al. investigated the effect of maghemite on
74 biohydrogen production via dark-photo fermentation and claimed that hydrogen
75 production could be remarkably enhanced with the addition of maghemite NPs by
76 promoting the bioactivity of hydrogen-producing microbes [11]. Gadhe et al. used
77 hematite and nickel oxide NPs to enhance the activity of ferredoxin oxidoreductase,
78 ferredoxin, and hydrogenase by accelerating electron transfer owing to the large
79 specific surface area and quantum size effects of NPs, which in turn stimulated
80 hydrogen production [12]. Beckers et al. studied the improving effects of conductive
81 metal (Pd, Ag, and Cu) and metal oxide (Fe_xO_y) on hydrogen fermentation using
82 *Clostridium butyricum*; these effects are mainly attributed to the enhanced activity of
83 hydrogenase and electron transfer rate to protons to generate molecular hydrogen [13].
84 Other conductive metal NPs, such as iron, nickel [14] and gold NPs [15], also can
85 enhance fermentative hydrogen production from carbohydrate. Zhang et al. reported

86 that adding appropriate concentrations of biochar could improve fermentative
87 hydrogen production from glucose by promoting the growth of hydrogen-producing
88 bacteria acting as carriers [16]. Granular activated carbon (GAC) can enhance
89 hydrogen fermentation by facilitating the formation of biofilm and efficient
90 colonization of microbes due to its large surface area [17] . Elreedy et al. noted that
91 biohydrogen production from industrial wastewater could be significantly promoted
92 by nickel-graphene nanocomposite because nickel NPs can provide metal nutrients for
93 the synthesis of [Ni-Fe] hydrogenase, whereas graphene substrate can enhance the
94 efficiency of electron transfer involved in hydrogen production [18]. However, the
95 scalable industrial production, cost, and quality are the most challenging obstacles for
96 the successful applications of these additives (such as metal NPs and graphene) in
97 hydrogen fermentation.

98 Carbon cloth is an electro-conductive material made of thousands of single
99 carbon fibers (diameter c. 6–10 μm) and has high chemical stability, electrical
100 conductivity, large surface area, and low production cost [19]. The large surface area
101 of carbon cloth facilitates the attachment and immobilization of microorganisms
102 because the carbon particles (diameter c. 1–2 mm) are larger than bacterial cells,
103 thereby offering sufficient attachment surfaces to the cells [20]. Similar to biochar
104 [16], carbon cloth could be favorable to enhance the attachment and growth of
105 hydrogen-producing microbes, thus serving as support carriers to improve
106 biohydrogen production. As an electro-conductive material, carbon cloth can facilitate

107 the potential electron communication between bacterial cells to reduce protons to
108 molecular hydrogen. However, its application in promoting hydrogen production is
109 poorly studied.

110 To date, the effective use of conductive carbon cloth on hydrogen production of
111 genetically modified *E. aerogenes* has not been reported yet. The microcosmic
112 characters of biofilms on carbon cloth have not been revealed. In this study, carbon
113 cloth was added to improve fermentative hydrogen production. The innovation and
114 objectives of this study are as follows: (1) compare hydrogen yield and production
115 rate with different additions of electro-conductive carbon cloth and non-conductive
116 cotton cloth in anaerobic digestion of glucose; and (2) analyze the compositions and
117 functional groups of the soluble microbial products (SMPs, mainly contain organic
118 macromolecules that are produced by microorganisms) in carbon cloth biofilm and
119 supernatant.

120

121 **2. Methods**

122 **2.1. Microorganisms, plasmids, and medium**

123 *E. aerogenes* ATCC13408 was purchased from China General Microbiological
124 Culture Collection Centre. The coding region of the *hoxEFUYH* genes were amplified
125 from the genome of *Synechocystis* sp. PCC 6803 with forward
126 (*hoxEFUYH*-F50-CCCGGGATGACCGTTGCCACCGAT-30) and reverse
127 (*hoxEFUYH*-R 50-CTCGAGCCATTGACATTGAGTTCTCC-30) primers. The

128 genetic modification method used was from a previous study [21], and the genetically
129 modified *E. aerogenes* was named *E. aerogenes/HoxEFUYH*. The bacteria were
130 cultivated in Luria Bertani (LB) culture medium which contained 5 g/L yeast extract,
131 10 g/L peptone, and 10 g/L NaCl. Solid LB medium also contained 20 g/L agar.

132 **2.2. Preparation of carbon cloth and cotton cloth**

133 Carbon cloth (Henghui Woven Carbon Fiber Co., China) was used as conductive
134 material. The electrical resistivity and surface area of carbon cloth are 0.0016 $\Omega\cdot\text{cm}$
135 and 8.4 m^2/g , respectively. During carbon cloth manufacturing, the surfaces of carbon
136 fiber are probably polluted by various organics, such as oils, alkaloid, and synthetic
137 resin [22]. Formic acid pretreatment was adopted to rinse the pollutants on the surface
138 of carbon cloth [19]. This process eliminated the nitrogen functional groups such as
139 pyrrole nitrogen and pyridine nitrogen, which hindered the electron transfer between
140 microorganisms and carbon cloth [23]. The raw cloth was cleaned with distilled water,
141 dried, and cut into circles of 2 cm in diameter. For formic acid modification, the raw
142 cloth was soaked in 5 mL of formic acid solution (mass fraction of 44%) for 12 h. The
143 cloth was then rinsed five times with distilled water and dried at 100 °C for 4 h in an
144 air atmosphere prior to subsequent experiments. The dried carbon cloth was
145 autoclaved at 121 °C for 15 min. For the control, non-electrical conductive cotton
146 cloth, with surface area and size equivalent to those of carbon cloth, was pretreated
147 similarly before being applied to reactors. The carbon cloth and cotton cloth were
148 subsequently added into the medium that contained *E. aerogenes/HoxEFUYH* under

149 anaerobic condition.

150 **2.3. Analysis methods**

151 **2.3.1. Scanning electron microscopy (SEM) analysis**

152 Electron micrographs of the carbon cloth were obtained under a field emission
153 SEM (Hitachi S3700, Japan) to visualize cell attachment to the carbon cloth.
154 Microorganisms with and without carbon cloth were observed separately. The digital
155 electron micrographs were captured with an accelerating voltage of 15 kV.

156 **2.3.2. Bicinchoninic acid method (BCA) analysis**

157 In brief, 1 mL of the culture medium was removed at 1 h and 96 h of
158 fermentation to quantify the protein content in the supernatant. The entire cloth was
159 separated from the liquid using tweezers to quantify the protein content in carbon
160 cloth and cotton cloth biofilms. Cell protein was extracted from the cloth by soaking it
161 in 1 mL of 0.2 N NaOH at 4 °C for 1 h and shaking every 10 s for 15 min. Afterward,
162 1 mL of deionized water was added to rinse the cloth, which was then removed. The
163 remaining liquid was frozen at -20 °C for 2 h and then placed in an electric-heated
164 thermostatic water bath at 90 °C for 10 min. The above procedure was repeated thrice.
165 The concentration of extracted protein was quantified using bicinchoninic acid
166 method with bovine serum albumin as the protein standard [24].

167 **2.3.3. Extraction and characterization of soluble microbial products**

168 Soluble microbial products (SMPs) mainly include proteins and polysaccharides.
169 The SMPs in carbon cloth biofilms were extracted. Carbon cloth was removed from *E.*
170 *aerogenes/HoxEFUYH* on the last day of fermentation. The biological membrane was

171 scraped off the surface and placed in a centrifuge tube. The carbon cloth was then
172 washed with deionized water to rinse the attached SMPs. The centrifuge tube
173 remained vibrating on a vortex generator for 10 min to crush the biofilm and
174 completely dissolve the SMPs. The mixture was then centrifuged at 7500 rpm for 10
175 min. The SMPs in supernatant were obtained using an acetate fiber microfiltration
176 membrane (0.45 μm).

177 A Fourier transform infrared spectra (FTIR, Nicolet 5700, USA) was used to
178 observe the difference in the chemical composition and functional groups between the
179 SMPs in carbon cloth biofilms and in supernatant. The SMPs were supplemented with
180 acetone and placed in the refrigerator at 4 °C for 24 h. The sediments were separated
181 and then vacuum dried at 60 °C for 12 h to obtain the dried SMP samples, which were
182 pulverized and then prepared using the potassium bromide (KBr) pellet method.
183 Infrared spectra were measured using a FTIR at room temperature. Each spectrum
184 was obtained from 400 cm^{-1} to 4,000 cm^{-1} [25].

185 The compositions of the SMP solution were characterized by fluorescence
186 excitation emission matrix spectra (EEM) using a Cary Eclipse fluorescence
187 spectrophotometer (Edinburgh Instruments, UK). The excitation wavelength was set
188 at 270 nm, whereas the emission wavelengths were detected from 280 nm to 600 nm
189 at 1 nm steps.

190 The particle sizes of SMPs in the biofilms were determined by
191 Zetasizer3000HSA nanolaser particle size analyzer (Malvern, UK) after the end of
192 fermentation. The particle size range was 2–3000 nm, and the test temperature was
193 20 °C. The principle is photon correlation spectroscopy. The liquid sample was

194 diluted to approach transparency and was ultrasonically treated for 3–5 min, and the
195 particle sizes were then tested.

196 **2.3.4. Gas chromatography analysis**

197 The gas produced from glucose fermentation mainly includes hydrogen, of which
198 the concentration was determined using gas chromatography (GC; Agilent 7820 A,
199 USA) system. GC conducted for 1 min using a 5A molecular sieve column and
200 HayeSep Q column at 65 °C. The temperature was then increased to 145 °C at the rate
201 of 25 °C/min for 1.8 min (argon flow rate was 27 mL/min).

202 Soluble metabolic degradation byproducts (SMDBs) that mainly contain acetate,
203 ethanol, propionate, isobutyrate, butyrate, isovalerate, valerate, and caproate were
204 analyzed using another GC system (Agilent 7820 A, USA) equipped with a flame
205 ionization detector and DB-FFAP column (Φ 0.32 mm \times 50 m, Agilent, USA). The
206 original temperature was maintained at 100 °C for 1 min and then increased to 200 °C
207 at the rate of 10 °C/min for 2.5 min.

208 **2.4. Calculations**

209 The hydrogen yields were simulated using the modified Gompertz equation (Eq.
210 1), whereas the dynamic parameters were calculated using the Origin 9.0 software.
211 Triplicate experiments for all the conditions were conducted to obtain mean values
212 and standard deviations.

$$213 \quad H = H_m \exp \left\{ -\exp \left[\frac{R_m e}{H_m} (\lambda - t) + 1 \right] \right\} \quad (1)$$

214 where H is the cumulative hydrogen yield, mL/g glucose; H_m is the maximum
215 hydrogen yield potential, mL/g glucose; R_m is the peak rate of hydrogen production,

216 mL/g glucose/h; and λ is the lag-phase time of hydrogen production, h.

$$217 \quad T_m = \frac{H_m}{R_m e} + \lambda \quad (2)$$

218 where T_m is the peak time, h.

219

220 **3. Results and discussion**

221 **3.1. Microscopic analysis of *E. aerogenes*/HoxEFUYH**

222 The cultures of *E. aerogenes*/HoxEFUYH in supernatant were observed via SEM.

223 As shown in Fig. 1, many nanowires were found between the bacteria in supernatant

224 without carbon cloth. Zhuang et al. found pili on the extracellular membranes of *E.*

225 *aerogenes*; these pili can serve as electric conduits for electron transfer in spite of

226 insufficient evidence (cyclic voltammograms of bacterial biofilm) [26, 27]. In this

227 study, scarcely any intercellular nanowires were observed after adding carbon cloth by

228 capturing more than 50 SEM images of *E. aerogenes*/HoxEFUYH added with carbon

229 cloth. The representative SEM images are shown as Fig. 1. The carbon cloth possibly

230 replaced pili to act as a conduit among bacteria cells for the electron transfer. The

231 electrical resistivity of carbon cloth is 0.0016 $\Omega \cdot \text{cm}$, which is relatively lower than

232 that of most microbial nanowires [28]. Thus, the microorganisms did not have to

233 produce nanowires, thereby reducing the energy consumption for growth.

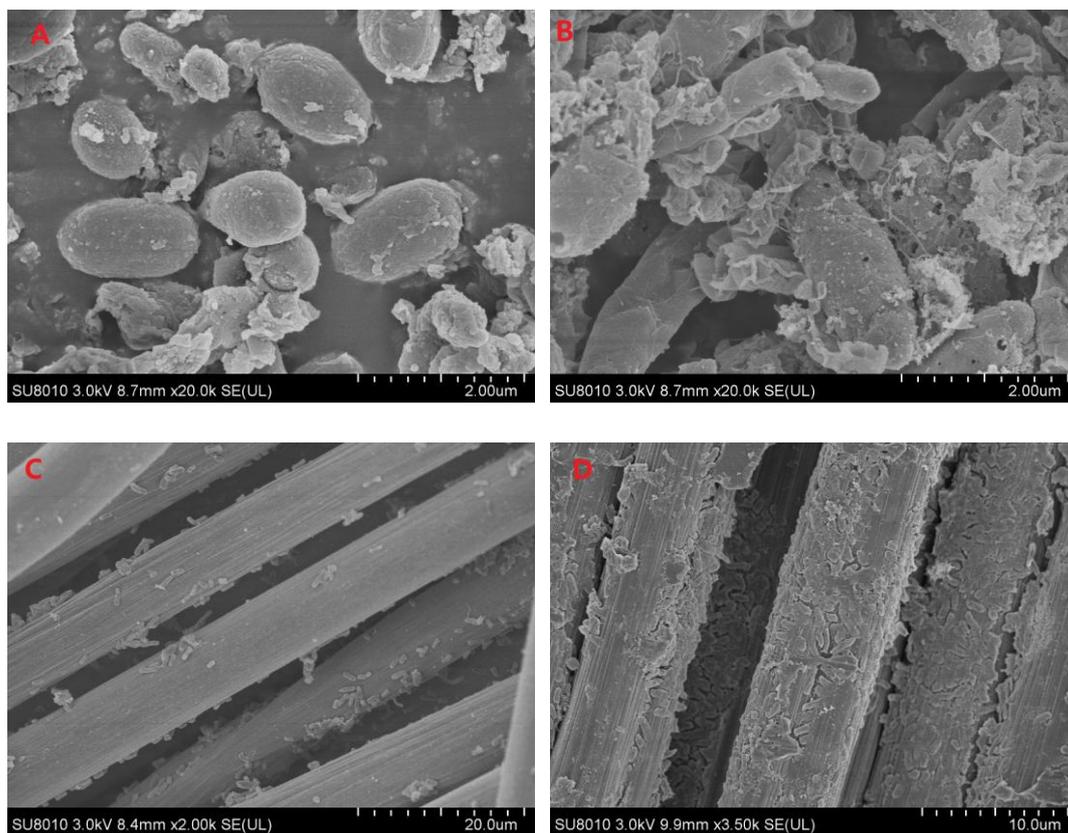
234 The SEM images of the carbon cloth on 1 h and 96 h of fermentation were

235 captured to investigate the surface changes in the carbon cloth. Initially, the surface of

236 carbon cloth was smooth and had few microorganisms. At the end of fermentation, the

237 surface was caked with microorganisms, and dense biofilms were formed. Similar

238 structural changes were reported in carbon nanotubes [29]. The carbon cloth provided
239 an attachment surface for *E. aerogenes/HoxEFUYH* in close contact with each other
240 and facilitated bacteria growth. Thus, the fermentation of glucose was accelerated
241 with the carbon cloth.



242

243 **Fig. 1.** SEM images of transgenic *E. aerogenes/HoxEFUYH* in the supernatant with/without
244 carbon cloth. (A) *E. aerogenes/HoxEFUYH* in the supernatant with carbon cloth at 20000 \times ; (B) *E.*
245 *aerogenes/HoxEFUYH* without carbon cloth at 20000 \times ; (C) Carbon cloth at 1 h of fermentation at
246 2000 \times ; (D) Carbon cloth at 96 h of fermentation at 3500 \times .

247 3.2. Analysis of biofilms

248 3.2.1. Protein contents analysis

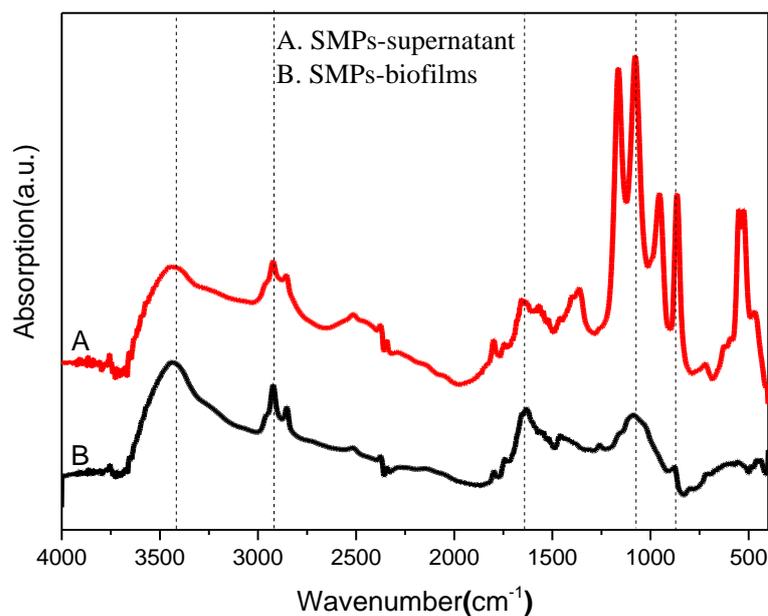
249 At 96 h of fermentation, approximately 59.1% of the microorganisms were
250 firmly attached to the solid particles of carbon fiber, of which the protein content was

251 approximately 2.6 g/L. By contrast, the protein content in supernatant was 1.8 g/L.
252 The protein content of biofilms attached to cotton cloth was measured as 2.5 g/L,
253 which indicated that the similar proportion of bacteria was immobilized on the cotton
254 cloth. Cell reproduction was promoted by the carbon cloth and cotton cloth because
255 they provided an improved growing environment for the cultures. In addition, the
256 carbon cloth probably substituted for the conducting microbial wires. The energy
257 consumed for microbial growth was reduced, which in turn accelerated the syntrophic
258 metabolism of glucose.

259 **3.2.2. FTIR analysis**

260 FTIR showed the similarities and differences between soluble microbial products
261 (SMPs) from carbon cloth biofilms and supernatant. As shown in Fig. 2, the peaks of
262 SMPs from the biofilms mainly appeared at 3415, 1620, and 1400 cm^{-1} . The
263 absorption bands at 3415 cm^{-1} (O-H) and 1400 cm^{-1} (C-N₂H) were from protein, and
264 that at 1620 cm^{-1} (C=O) was from polysaccharides [30]. Thus, the SMPs in carbon
265 cloth biofilms mainly included large quantities of protein and a small fraction of
266 polysaccharides.

267 Significant similarities existed between the SMPs in the supernatant and biofilms.
268 In the SMPs from the supernatant, three peaks existed between 800—1200 cm^{-1} ,
269 which were ascribed to the C-O stretching vibration in the polysaccharide compound.
270 This finding indicated that polysaccharides occupied a large part of the SMPs in the
271 supernatant as a result of the metabolic substance released from the microorganism.
272 Some deviations existed in peak positions; thereby indicating SMP compositions in
273 the biofilms and supernatant were different.



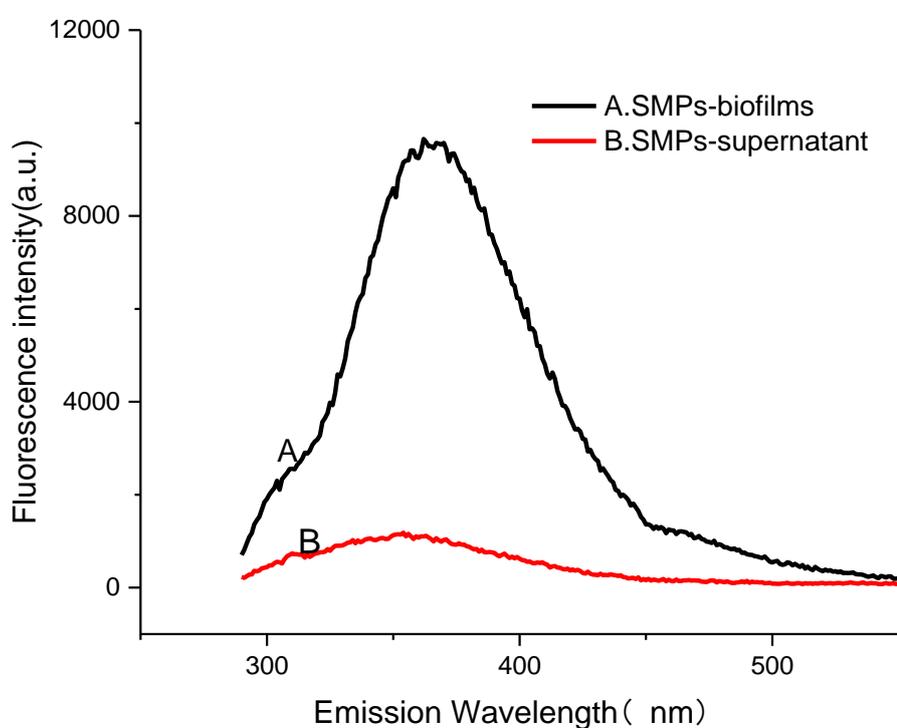
274

275 **Fig. 2.** Fourier transform infrared spectra of soluble microbial products in carbon cloth biofilms
 276 and supernatant.

277 3.2.3. EEM analysis

278 EEM spectra of SMPs in carbon cloth biofilms and supernatant were tested after
 279 fermentation. The results in Fig. 3 reveal that the SMPs in biofilms and supernatant
 280 both showed peaks. Compared with supernatant SMPs, biofilm SMPs presented
 281 higher intensity peaks, indicating that both of them contained organic matter that
 282 could be emitted by fluorescence. However, the organic matter content in biofilm
 283 SMPs was higher than that in supernatant. The peak area of fluorescence spectrum
 284 can roughly indicate the relative content of fluorescent substances in SMPs. Through
 285 peak group analysis and calculation with Origin 9.0, the relative content of fluorescent
 286 substances in carbon cloth biofilm SMPs (88.1%) was found to be larger than that in
 287 supernatant SMPs (11.9%). In the EEM diagram of the carbon cloth biofilm SMPs,

288 the location of the peak was concentrated in 357–374 nm, which was associated with
289 soluble microbial by-product-like and humic substance-like substances according to
290 the classification scheme by Chen et al. [31, 32]. Although carbon cloth biofilm SMPs
291 and supernatant SMPs both contained protein substances, their peak positions were
292 different, indicating that the two kinds of proteins contained different species. This
293 result was consistent with FTIR.

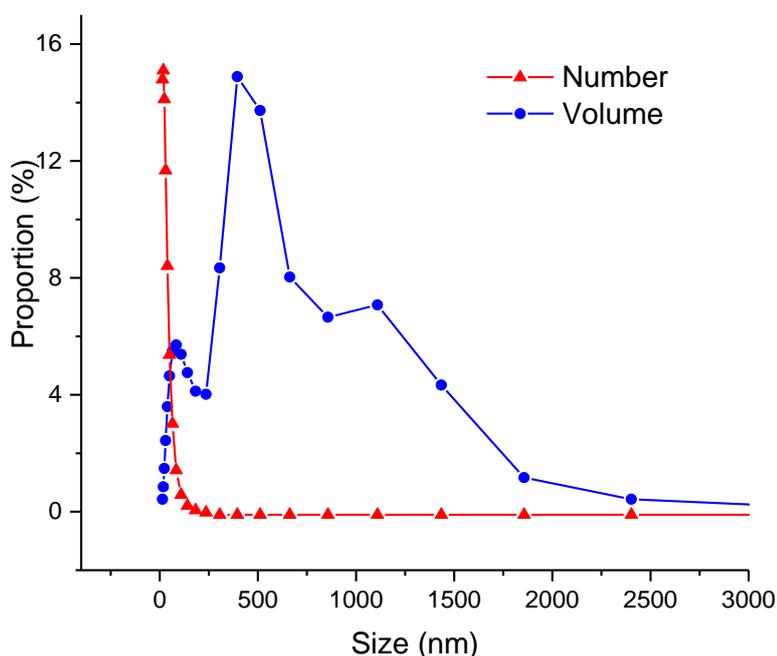


294
295 **Fig. 3.** Excitation Emission Matrix spectra analysis of soluble microbial products in carbon cloth
296 biofilms and supernatant.

297 **3.2.4. Particle size analysis of biofilm SMPs**

298 During fermentation, the organic matter of carbon cloth biofilm SMPs can
299 diffuse into supernatant, thereby promoting the mass transfer between biofilm and

300 supernatant. The diffusion of macromolecules from biofilm SMPs to supernatant
301 strictly depends on the particle size. The particle sizes of biofilm SMPs at the end of
302 fermentation were determined by volume and quantity as shown in Fig. 4. Different
303 measurement methods showed different particle size distributions. With the volume as
304 the index, the particle size distribution showed three peaks. Molecules from 14 nm to
305 1000 nm occupied 85.6% of the total particles. Three peaks appeared at 84.3, 395.5,
306 and 1108.8 nm, and the average particle size was 465.2 nm. With the index of number,
307 the peak only appeared at 18 nm. Approximately 98.4% of the molecular sizes were
308 concentrated in 12–200 nm, and the average particle size was 30.2 nm.



309
310 **Fig. 4.** Particle size distribution of soluble microbial products in carbon cloth biofilms.
311 The molecular sizes of the carbon cloth biofilm SMPs were less than 1.2 μm .
312 The diameter of carbon cloth fiber is 6.9 μm , and the particle sizes of biofilm SMPs
313 were smaller than those of carbon fiber. This finding indicated that during

314 fermentation, the substance in biofilm could easily diffuse into the supernatant. These
315 matter and energy flow processes facilitated the hydrogen production by *E.*
316 *aerogenes/HoxEFUYH*.

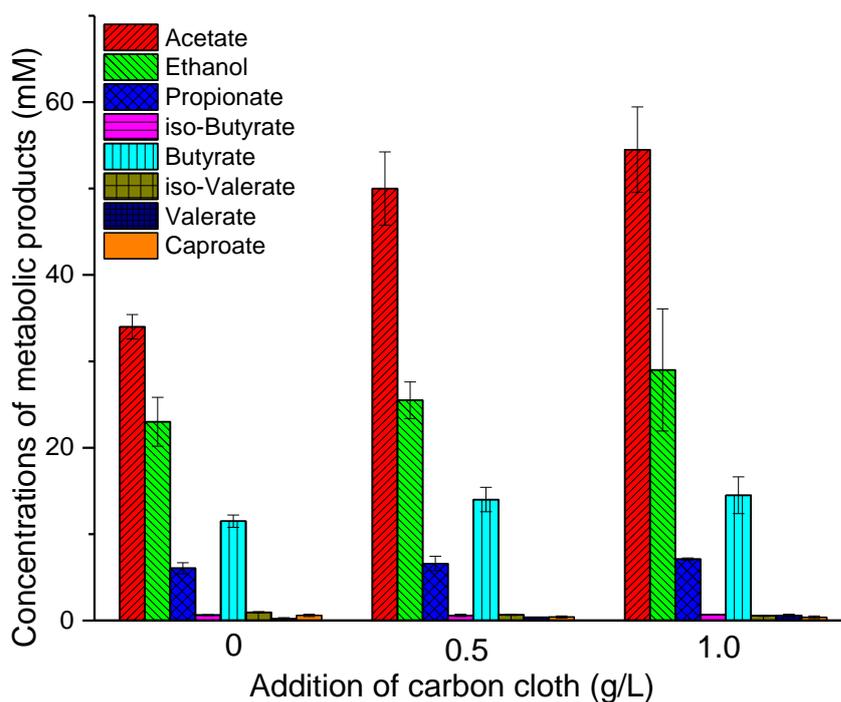
317 **3.3. Soluble metabolic degradation products analysis**

318 The total soluble metabolic degradation byproducts (SMDBs) of the dark
319 hydrogen fermentation effluent are shown in Fig. 5. SMDBs included acetate, ethanol,
320 propionate, butyrate, isobutyrate, isovalerate, valerate, and caproate. The acetate (34
321 mM to 54.5 mM) and ethanol (23 mM to 29 mM) made up most of the liquid products.
322 With 1.0 g/L carbon cloth, the SMDBs increased from 76.9 mM to 107.3 mM. The
323 proportion of ethanol in SMDBs decreased from 29.9% to 27.0%; nevertheless, the
324 proportion of acetate in SMDBs increased from 44.2% to 50.8%. As shown in Eqs. 3
325 and 4 [33], ethanol pathway theoretically cannot produce hydrogen, whereas acetate
326 pathway can produce 4 mol hydrogen. The metabolic pathways of *E.*
327 *aerogenes/HoxEFUYH* were changed by accessing suitable carbon cloth, which led to
328 increased acetate and less ethanol levels. The hydrogen production increased
329 correspondingly.



332 The share of propionate in SMDBs decreased from 7.9% to 6.6%. During
333 glucose degradation, pyruvate was first produced through glycolysis; two different
334 metabolic pathways were reported for pyruvate [7]. One is the propionate pathway, in
335 which pyruvate was directly decomposed to propionate without hydrogen production.

336 The other is the formate pathway, in which pyruvate was degraded into formate,
 337 which was then decomposed to hydrogen. The reduction of propionate suggested that
 338 proper additions of carbon cloth possibly enhanced the formate pathway and
 339 restrained the propionate pathway. As a result, additional hydrogen was obtained.



340

341 **Fig. 5.** Soluble metabolic degradation byproducts in hydrogen fermentation of glucose added

342

with carbon cloth.

343 **3.4. Fermentative hydrogen production from glucose**

344 Hydrogen yield (191.3 ± 2.34) was relatively low without any additives. After

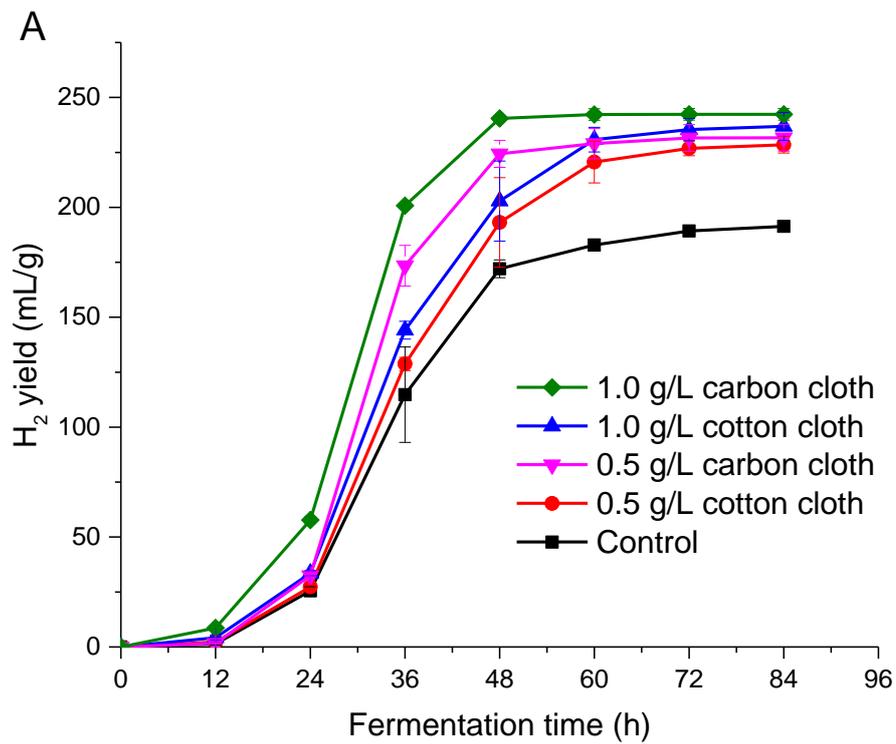
345 adding 0.5 and 1.0 g/L carbon cloth, the hydrogen yield reached to 231.7 ± 6.12 and

346 242.3 ± 2.65 mL/g glucose, respectively (Fig. 6). The highest hydrogen production

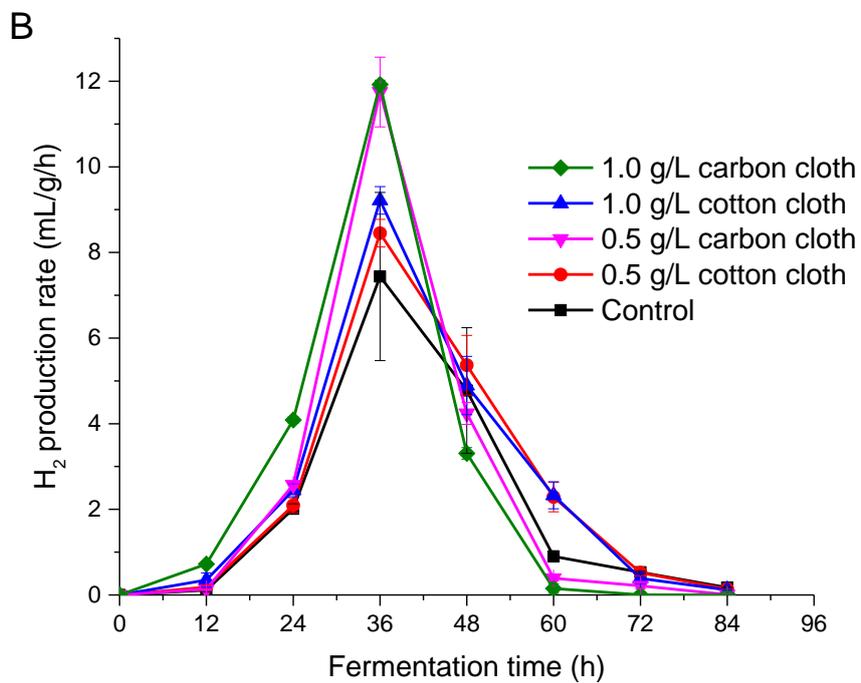
347 rate of 11.9 ± 0.10 mL/g/h was achieved in the presence of 1.0 g/L carbon cloth at 36

348 h. The hydrogen yield also increased to 228.5 ± 3.77 and 236.9 ± 6.35 mL/g glucose
349 in the presence of 0.5 and 1.0 g/L cotton cloth, respectively. The carbon cloth and
350 cotton cloth are favorable to the attachment of microorganisms acting as supporting
351 materials and facilitate bacteria growth. As indicated by the analysis of protein
352 contents, the number of microorganisms attached to the surface of carbon or cotton
353 cloth was higher than that in the supernatant. In addition, the enhancement effect on
354 hydrogen production rate of carbon cloth is more evident than of cotton cloth. A
355 possible reason is the carbon cloth can assist the potential electron transfer among
356 hydrogen-producing bacteria, thereby accelerating the metabolism of glucose to
357 hydrogen. Carbon cloth possibly served as electron conduits among bacteria, thus
358 eliminating the need for biological connections such as nanowires. This result was
359 consistent with a previous study, which confirmed that carbon cloth could promote
360 electron transfer [34].

361 Triplicate experiments for all the conditions were conducted to obtain the
362 average data and their standard deviations. In Fig. 6, error bars represent ± 1 standard
363 deviation of triplicates. Experimental data were evaluated by analysis of variance
364 (ANOVA) applying Tukey test in Origin software at a significance level of 0.05. The
365 P values (Prob>F) are 0.025 and 0.009 in hydrogen yield and hydrogen production
366 rate, which indicate that the differences of results are statistically significant.



367



368

369 **Fig. 6.** H₂ production of transgenic *E. aerogenes*/HoxEFUYH with carbon cloth and cotton cloth

370

from glucose. (A) H₂ yield; (B) H₂ production rate.

371

Table 1. Dynamic parameters of fermentative H₂ production of transgenic *E.*

372

aerogenes/HoxEFUYH with carbon cloth and cotton cloth from glucose.

| Additive | H ₂ yield (mL/g) | H ₂ production peak rate (mL/g/h) | Dynamic parameters | | | | |
|----------------------------|---------------------------------|---|--------------------------------|--------------------------------------|------------------|-----------------------------|-----------------------|
| | | | <i>H_m</i> (mL/g) | <i>R_m</i> (mL/g /h) | λ (h) | <i>T_m</i> (h) | <i>R</i> ² |
| Control | 191.3 ± 2.34 | 7.44 ± 1.97 | 190.9 | 8.3 | 21.6 | 30.1 | 0.9996 |
| 0.5 g/L cotton cloth | 228.5 ± 3.77 | 8.45 ± 0.32 | 229.6 | 9.0 | 21.6 | 31.0 | 0.9999 |
| 1.0 g/L cotton cloth | 236.8 ± 6.35 | 9.22 ± 0.32 | 237.5 | 9.6 | 20.8 | 30.0 | 0.9995 |
| 0.5 g/L carbon cloth | 231.7 ± 6.12 | 11.7 ± 0.82 | 231.8 | 13.7 | 22.0 | 28.2 | 0.9999 |
| 1.0 g/L carbon cloth | 242.3 ± 2.65 | 11.9 ± 0.10 | 243.3 | 15.2 | 20.3 | 26.2 | 0.9990 |

373

The kinetic parameters of hydrogen production fitted by the modified Gompertz

374

equation are shown in Table 1. The kinetics of hydrogen production was evaluated in

375

terms of hydrogen yield potential (*H_m*), peak hydrogen production rate (*R_m*), lag phase

376

time (λ), and peak time (*T_m*). The maximum hydrogen yield potential (*H_m*, 243.3 mL/g)

377

was achieved in the presence of 1.0 g/L carbon cloth, corresponding to a value of 27.4%

378

higher than the control. The lag phase time (λ) and peak time (*T_m*) were both reduced

379

in the presence of 1.0 g/L carbon cloth. The carbon cloth effectively promoted

380

hydrogen production using *E. aerogenes/HoxEFUYH*. This result was consistent with

381

a previous study, which reported that the hydrogen yield increases with the addition of

382 biochar [35].

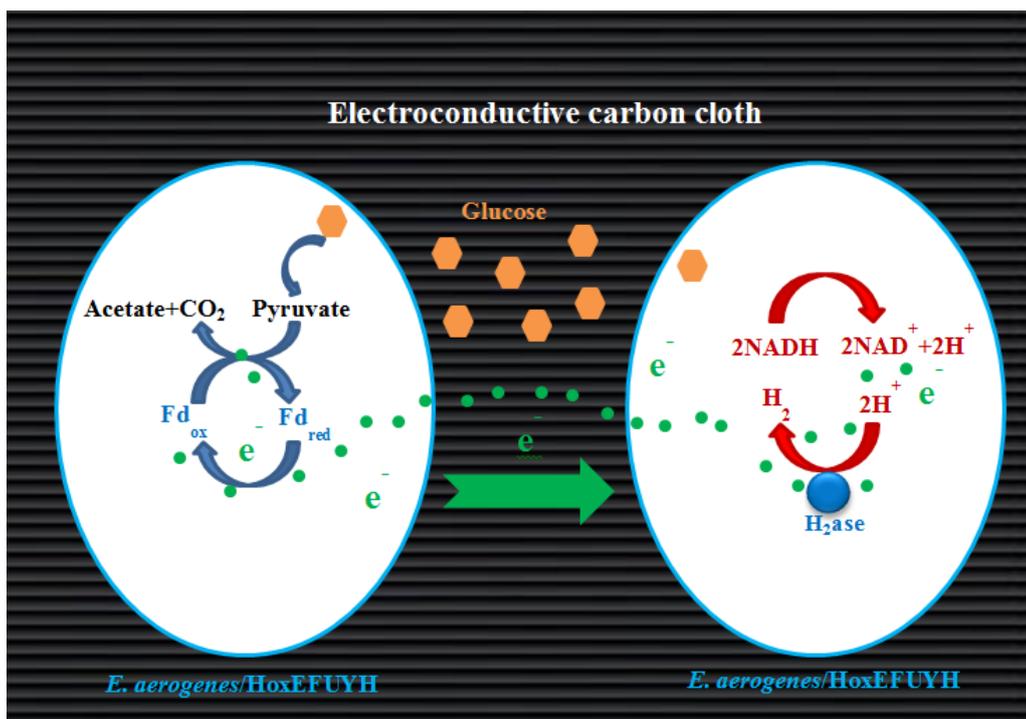
383 **3.5. Proposed mechanisms for enhanced hydrogen production with carbon** 384 **cloth**

385 As illustrated in Fig. 1, dense microorganism films were formed on the surface of
386 carbon cloth at the end of hydrogen fermentation since the carbon cloth with large
387 surface area is capable of providing support carriers for the attachment and
388 immobilization of bacteria. Furthermore, the analysis of protein content and EEM
389 spectra indicated that microbes attached to the surface of carbon cloth were much
390 denser than those suspended in the supernatant. This kind of cell immobilization
391 technique belongs to surface-attached biofilms [36, 37]. It has been reported that
392 attached cell immobilization is more superior to suspended cell since the fermentation
393 system is more likely to maintain process stability and higher microbial activity [17].
394 Moreover, the character of porous structure of carbon cloth is favorable of sustaining
395 cell viability, avoiding excess leakage of bacteria and promoting bacterial colonization
396 [17, 36, 38]. Therefore, carbon cloth added in the fermentation system is conducive to
397 create high cell density and efficient hydrogen production.

398 Apart from having the function of cell immobilization and facilitating biofilm
399 formation, carbon cloth may be capable of promoting the potential bacterial electron
400 transfer. Based on the SEM images of *E. aerogenes/HoxEFUYH* cultures with carbon
401 cloth (Fig. 1), the bacterial nanowires in the supernatant cells almost disappeared with
402 the addition of carbon cloth. The potential approach of electron transfer between cells

403 with nanowires replaced by carbon cloth can be changed. Given that the electrical
404 resistivity of carbon cloth ($0.0016 \Omega \cdot \text{cm}$) is lower than that of most microbial
405 nanowires ($\sim 0.5 \Omega \cdot \text{cm}$) [28], the electron transport through conductive carbon cloth is
406 likely to be more efficient than through intercellular nanowires.

407 Bacterial communities can exchange cytoplasmic factors, such as proteins [39],
408 nutrients [40], and electrons [41] via intercellular membrane nanotubes. Dubey et al.
409 proposed that nanotube-like membrane structures are major channels of bacterial
410 communication in nature; these channels can offer conduits for exchange of
411 cytoplasmic molecules within and between species [42]. In the current work, the
412 nanowire-like connections between *E. aerogenes*/HoxEFUYH cells possibly serve as
413 communication channels to exchange electrons. As exoelectrogenic bacteria, *E.*
414 *aerogenes* have been applied in the field of microbial fuel cells (MFCs) for a long
415 time; previous studies indicated that the current generation is not only attributed to
416 situ biohydrogen oxidization but is also related to direct electroactive biofilms [6].
417 Reguera et al. also reported that *E. aerogenes* might directly transfer electrons to the
418 electrode via the electroconductive pili on their external membranes in biofilms [43].
419 In summary, the electron exchange between neighboring cells via conjugative
420 nanowires is a possible form of bacterial communication.



421

422 **Fig. 7.** Schematic diagram of the proposed mechanism for enhancing electron transfer between

423 *transgenic E. aerogenes/HoxEFUYH* cells with conductive carbon cloth.

424 In this study, many bacterial nanowires were observed in the SEM images of *E.*

425 *aerogenes/HoxEFUYH* without carbon cloth (Fig. 1B). These nanowires can serve as

426 electric conduits for the electron transfer among *E. aerogenes/HoxEFUYH* cells.

427 Almost no bacterial nanowires were observed in the supernatant cells due to the

428 presence of conductive carbon cloth (Fig. 1A). As illustrated in Fig. 7, it is likely that

429 the carbon cloth replaced nanowires to act as a conduit among bacteria for the

430 electron transfer. The primary hydrogen synthesis process in *E.*

431 *aerogenes/HoxEFUYH* cell could be characterized as follows: glucose degraded to

432 pyruvate, which was converted to acetylCoA, which can be further decomposed into

433 acetate, and ferredoxin can be reduced by NADH: ferredoxin oxidoreductase (NFOR)

434 [3, 44]. In view of the potential interactions and electron transport within *E. aerogenes*

435 species, the electrons released from reduced ferredoxin are likely to be transferred
436 across the cell membrane to the hydrogenase in an adjacent cell through carbon cloth.
437 This process aims to reduce the protons provided by NADH to molecular hydrogen, in
438 case the ferredoxin could not instantly feed sufficient electrons to hydrogenase in the
439 electron-accepting cell. The rate of intercellular electron transfer is likely to be
440 enhanced with carbon cloth because the electrical resistivity ($0.0016 \Omega \cdot \text{cm}$) of which
441 is lower than that of most microbial nanowires [28]. Thus, hydrogen production rate
442 increased with proton reduction efficiency. In summary, the carbon cloth potentially
443 promoted hydrogen production by accelerating the electron transfer among *E.*
444 *aerogenes*/HoxEFUYH cells.

445

446 **4. Conclusions**

447 This study reported that conductive carbon cloth can facilitate dark hydrogen
448 fermentation potentially due to the enhancement of intercellular electron transfer
449 among *E. aerogenes* cells. Carbon cloth provided a sufficient place for bacterial
450 attachment, which led to the biofilm growth with 59.1% of the total microorganism
451 population. SEM showed the disappearance of bacterial nanowires, suggesting that
452 carbon cloth possibly replaced bacterial nanowires to serve as electron conduits. FTIR
453 analysis revealed that SMPs in carbon cloth biofilms mainly contained
454 polysaccharides, proteins, and humic-like substances. EEM indicated that the relative
455 content of fluorescent substances in biofilm SMPs (88.1%) is larger than that in

456 supernatant SMPs (11.9%). Metabolic analysis revealed that carbon cloth stimulated
457 acetate pathway but impaired ethanol pathway. While this study displays the
458 feasibility of using conductive carbon cloth to promote hydrogen fermentation, the
459 mechanism of bacterial communication and electron transfer in the presence of carbon
460 cloth need to be further investigated.

461

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