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1 **Optimization of key factors affecting biohydrogen production from microcrys-**
2 **talline cellulose by co-culture of** *Clostridium acetobutylicum* X_9+ *Ethanoigenens*
3 *harbinense* B_2

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21 **Abstract**

22 Key factors (initial pH value, substrate concentration, incubation time, C/N,
23 L-cysteine concentration) affecting biohydrogen production from microcrystalline
24 cellulose in batch fermentation by co-culture of isolated strains (*Clostridium acetobu-*
25 *tylicum* X_9 + *Ethanoigenens harbinense* B_2) were optimized using an orthogonal ex-
26 periment. The isolated strain *Clostridium acetobutylicum* X_9 had high hydrogen yield
27 from microcrystalline cellulose (MCC), and *Ethanoigenens harbinense* B_2 could pro-
28 duce hydrogen efficiently from monosaccharide directly from microcrystalline cellu-
29 lose. The optimal parameters were as follows: 6.0 of initial pH value, 12 g L^{-1} of sub-
30 strate concentration, 40 h of incubation time, 0.7 g L^{-1} of L-cysteine concentration and
31 4:1 ratio of C/N. Under the optimum culture conditions, a maximum hydrogen yield
32 rate of 10.4 mmol g-MCC^{-1} was obtained. This yield was approximately 2.2-fold
33 greater than that of mono-culture *Clostridium acetobutylicum* X_9. It suggests that the
34 optimal conditions achieved can be applied to produce hydrogen from microcrystal-
35 line cellulose using co-culture of isolated strains *Clostridium acetobutylicum* X_9 +
36 *Ethanoigenens harbinense* B_2.

37

38 ***Key words***: Biohydrogen; Co-culture; Microcrystalline cellulose; Orthogonal ex-
39 periment

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40 **1 Introduction**

41 Energy is an essential commodity for increasing productivity in both agriculture and
42 industry. The worldwide energy demand has been increasing rapidly. It has serious
43 negative effects on environment under energy crisis and global warming.[1, 2] As a re-
44 newable energy, biomass power has had a rapid growth in the past decade in China.[3, 4]
45 Biomass will play an important role in global energy infrastructure in the future for
46 the generation of power and heat, along with the production of chemicals and fuels.[5]
47 Over the years, many researchers have already studied some biomass conversion to
48 energy, such as hydrogen, ethanol and methane.[6-10]

49 Currently, hydrogen is produced, exclusively, by electrolysis of water or by steam
50 reformation of methane. Biological technologies of hydrogen production provides a
51 wide range of approaches to generate hydrogen.[11] Biological hydrogen production
52 from renewable lignocellulosic waste has attracted significant attention.[12] The study
53 of Alibardi and Cossu indicated that the bread–pasta fraction in organic waste had a
54 marked effect on hydrogen potential production.[13] Ren et al. presented a comprehen-
55 sive review on the bioconversion of lignocellulosic biomass to hydrogen, which sheds
56 light on the perspectives on the lignocellulosic biomass conversion to hydrogen.[14] To
57 generate hydrogen directly from lignocellulose materials by using dark fermentation
58 requires expensive pretreatment processes for release underlying monomeric sugars,
59 such as delignification and hydrolysis.[15-17] Therefore, prior to DF, these biomasses are
60 often subjected to physical, chemical and biological pre-treatment to increase their
61 digestibility.[18] Favaro et al. reported that a properly pre-treated inoculum could be

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62 used to improve hydrogen H_2 yield from organic waste.[19] Microcytalline cellulose
63 (MCC) is cellulose derived from high quality wood pulp by acid hydrolysis to remove
64 the amorphous regions.[20] It is a purified partially depolymerized non-fibrous form of
65 cellulose that occurs as a white, odorless, tasteless, crystalline powder composed of
66 porous particles.[21] Therefore, microcrystalline cellulose (MCC) can be effectively uti-
67 lized as a model substrate to produce hydrogen.

68 The co-culture of cellulolytic and hydrogen-producing strains, by taking advantage
69 of their specific metabolic capacities, offers a promising new way to enhance the
70 conversion efficiency of cellulose to hydrogen.[22] Many studies about co-culture have
71 investigated for enhancing hydrogen production.[12, 23, 24] For improving the hydrogen
72 production efficiency, two aspects can be considered. Both characterization of the key
73 factors affecting biohydrogen production by co-culture strains and identification of
74 the ecological relationship among the organisms will contribute it.

75 Based on this background, the aim of this work was to explore the optimal condi-
76 tions of key factors affecting biohydrogen production by co-culture of isolated strains
77 *Clostridium acetobutylicum* X_9 + *Ethanoigenens harbinense* B_2. In order to find an
78 optimal combination of factor levels, single-factor experiment and orthogonal exper-
79 iment were used in this experimental design, and series of experiments were con-
80 ducted.[25]

81 **2 Materials and Methods**

82 2.1. Hydrogen-producing strains

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83 The strains *Clostridium acetobutylicum* X_9 (NCBI: EU434651) and *Ethanoigenens*
84 *harbinense* B_2 (NCBI: EU639425) were isolated from activated sludge in a pilot-scale
85 continuous fermentative hydrogen production reactor (working volume: 1.48 m^3, sub-
86 strate: molasses). The operation was conducted under organic loading rates of 3.11–
87 85.8 kg COD $m^{-3}d^{-1}$ for over 200 days.[26] Strain *Clostridium acetobutylicum* X_9 had
88 typical butyrate-type fermentation metabolism with high hydrogen yield and cellulose
89 degradation, whereas *Ethanoigenens harbinense* B_2 which has a 98% similarity to B_{49}
90 underwent so-called ethanol-type fermentation metabolism with high hydrogen yield.
91 [27]

92 2.2. Batch experiments

93 Batch experiments were carried out anaerobically in 100 mL or 250 mL serum bottles
94 at 37~40 ºC and operated in an orbital shaker at a rotation speed of 90~130 r min^{-1}.
95 The fermentation broth composition was (g L^{-1}): microcrystalline cellulose 12, pep-
96 tone 4.0, beef extract 2.0, yeast extract 1.0, NaCl 4.0, K_2HPO_4 1.0, $MgCl_2$ 0.1, $FeSO_4$
97 0.1 and L-cysteine 0.5. Moreover, 10 ml L^{-1} medium of vitamins (cyanocobalamin
98 0.01 g L^{-1}, ascorbic acid 0.025 g L^{-1}, riboflavin 0.025 g L^{-1}, citric acid 0.02 g L^{-1}, pyr-
99 idoxin 0.05 g L^{-1}, folic acid 0.01 g L^{-1}, 4-aminobenzoic acid 0.01 g L^{-1}, and creatine
100 0.025 g L^{-1}) and micronutrients ($MnSO_4·7H_2O$ 0.01 g L^{-1}, $ZnSO_4·7H_2O$ 0.05 g L^{-1},
101 H_3BO_3 0.01 g L^{-1}, $N(CH_2COOH)_3$ 4.5 g L^{-1}, $CaCl_2·2H_2O$ 0.01 g L^{-1}, Na_2MoO_4 0.01 g
102 L^{-1}, $CoCl_2·6H_2O$ 0.2 g L^{-1}, $AlK(SO_4)_2$ 0.01 g L^{-1}) were added. And 1 mL of resazurin
103 (0.2%) was also added to verify whether the reaction system was in the anaerobic

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104 condition. The microcrystalline cellulose had cellulose content at 97.2% (v/v, dry ba-
105 sis) and a water solubility of 0.1% (w/v).

106 In the co-culture experiments, the two strains *Clostridium acetobutylicum* X_9 +
107 *Ethanoigenens harbinense* B_2 were mixed at the same volumes at a total biomass
108 quantity of 6 mL (6%). All the experiments are repeated three times.

109 2.3 Analytical methods

110 In fermentative experiments, hydrogen production, end liquid products, cellulose
111 degradation, quantities of reductive saccharides were measured.

112 A gas chromatography (SCⅡ, Shanghai Analytical Apparatus, China) was used to
113 determine hydrogen content in the gas phase with a thermal conductivity detector
114 (TCD) and nitrogen as the carrier gas (70 mL min^{-1}). A 2.0-m stainless column was
115 packed with TDS-01 (60-80 meshes). The column and detector were all kept at 150ºC.

116 Another gas chromatography (GC122, Beijing Oriental Fine Hua Yuan Co., China)
117 was employed to detect the volatile fatty acids (VFAs) and alcohol contents in cen-
118 trifugated (4000r min^{-1}) fermenting liquor with nitrogen as the carrier gas (flow rate
119 of 60 mL min^{-1}). The hydrogen flame-ionization detector (FID) was employed with
120 hydrogen and air flow rates of 50 and 490mL min^{-1}, respectively, and a 2.0-m
121 GDX-103 (60-80 meshes) column. The column and all cells were kept at 190 ºC.

122 Microcrystalline cellulose centration was determined by phenol–H_2SO_4 method af-
123 ter removal of cell mass as described by Minato et al.[28] The amount of reducing sugar
124 was determined by the DNS method using xylose (Sigma) as a standard. One unit (U)

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125 of xylanase activity was expressed as 1 μ mol of reducing sugar (xylose equivalent)

126 released in 1 min.[29]

127 Based on the data of cumulative H_2 production and MCC substrate content in batch

128 experiment, the H_2 yield and cellulose degradation were calculated as following:

129 $$H_2 \text{ yield} = n_{H_2} / (M_1 - M_2) \quad (1)$$

130 $$\text{Cellulose degradation} = \frac{M_1 - M_2}{M_1} \times 100\% \quad (2)$$

131 In which n_{H2} (mmol) is maximum cumulative H_2 production, M_1(g) is MCC sub-

132 strate content before fermentation, and M_2 (g) is the MCC substrate content after fer-

133 mentation. If it is not marked, the data was measured at 40 h after the inoculation.

134 **3 Results and discussion**

135 3.1. Single-factor experiment

136 3.1.1. Effects of initial pH

137 Initial pH was important in increasing the efficiency of cellulose hydrolysis and sig-

138 nificantly affected the cumulative hydrogen production.[30, 31] Fig. 1 (a) and Fig1. (c)

139 show that hydrogen yield and cell dry weight respectively increased with the increas-

140 ing in initial pH from 3.0 to 6.0 and then decreased when the initial pH was greater

141 than 6.0. At initial pH 6.0, the maximum H_2 yield of *Clostridium acetobutylicum* X_9 +

142 *Ethanoigenens harbinense* B_2 reached 9.63 mmol g-MCC^{-1}, and cellulose degradation

143 was 81%. Besides, the cell dry weight reached 0.66 g L^{-1}. And Fig. 1 (b) shows that

144 the maximum concentrations of ethanol, butyrate and acetate in the end liquid prod-

145 ucts were 2166 mg L^{-1}, 1483 mg L^{-1} and 1994 mg L^{-1}, respectively. Additionally, the

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146 reducing sugar in different initial pH value did not accumulate to a detectable quantity
147 in the fermenting liquid.
148 At initial pH 6.0, the molar ratio (ethanol/butyrate/acetate) of the end liquid prod-
149 ucts was nearly 1.5:1.0:1.5. In other words, co-cultured strains underwent the etha-
150 nol-type fermentation researched by Ren et al.[32] High content of ethanol in end liquid
151 products could buffer ferment-end pH and reduce the inhibition of end products from
152 MCC, which was beneficial for maintaining the stability of microbial growth and hy-
153 drogen formation.
154 3.1.2. Effects of substrate concentration
155 Substrate concentration had individual significant influences on optimizing hydrogen
156 yield. [13, 33] Hence, the effect of the substrate concentration on biohydrogen production
157 should be revealed. As shown in Fig. 2 (a), (b) and (c), hydrogen yield, cellulose deg-
158 radation, end liquid products and cell dry weight increased with the increase in sub-
159 strate concentration from 1.0 g L^{-1} to 12.0 g L^{-1}. At substrate concentration of 12.0 g
160 L^{-1}, the maximum H_2 yield of *Clostridium acetobutylicum* X_9 + *Ethanoigenens har-*
161 *binense* B_2 reached 10.2 mmol g-MCC^{-1}, cellulose degradation of 86%, the cell dry
162 weight of 0.64 g L^{-1}, and the maximum concentrations of ethanol, butyrate and acetate
163 in the end liquid products were 2320 mg L^{-1}, 1520 mg L^{-1} and 1949 mg L^{-1}, respec-
164 tively. Similarly, the reducing sugar did not accumulate to a detectable quantity in the
165 fermenting liquid.
166 Understanding the dependence of substrate concentration on fermentative hydrogen
167 production is a critical step toward optimal control. Whether substrate concentration is

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168 moderate or not, it will directly affect the state of the growth and the activation of hy-
169 drogen-producing bacteria. That is to say, too high or too low substrate concentration
170 will affect related enzyme secretion and metabolic pathways of bacteria hydrogen
171 production.

172 3.1.3. Effects of incubation time

173 Sreela-or et al.[34] and Lo et al.[35] discussed the effects of incubation time on hydrogen
174 production, enzyme activity and reducing sugar production. Fig. 3 (a) and (b) reveal
175 that hydrogen yield, cellulose degradation and end liquid products increased from 26
176 h (mid-late log phase) to 40 h and achieved a steady state after 40 h of incubation time.
177 At incubation time 40 h, the maximum H_2 yield of *Clostridium acetobutylicum* X_9 +
178 *Ethanoigenens harbinense* B_2 reached 10.2 mmol g-MCC^{-1}, cellulose degradation of
179 85%. The cell dry weight reached steady after incubation about 40 h which leaded to
180 the maximum ethanol, butyrate and acetate concentrations of 2387 mg L^{-1}, 1510 mg
181 L^{-1} and 2069 mg L^{-1}, respectively, in the end liquid products. Likewise, the reducing
182 sugar did not accumulate to a detectable quantity in the fermenting liquid. Fig.3 (c)
183 shows that the cell dry weight increased from 26h to 40h. At 40h, multiple microor-
184 ganisms came to a stable phase.

185 3.1.4. Effects of C/N

186 Carbon and nitrogen are needed for the growth and metabolism of microorganisms. A
187 proper C/N ratio could enhance the material metabolized and bacterial hydrogen pro-
188 duced.[36, 37] In this experiment, microcrystalline cellulose (MCC) was used as sole

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189 carbon source, and yeast extract/peptone/beef extract (1:1:1) was used as the complex
190 nitrogen source.

191 Fig. 4 (a) , (b) and (c) illustrate that the hydrogen yield, cellulose degradation, end
192 liquid products and cell dry weight increased with increase in C/N ratio ranging from
193 0 to 4.0. At C/N ratio 4.0, the maximum H_2 yield rate of *Clostridium sp.* X_9 + *Etha-*
194 *noigenens harbinense* B_2 reached 10.26 mmol g-MCC^{-1}, cellulose degradation of 86%,
195 the dry cell was 0.72 g L^{-1}, and the maximum concentrations of ethanol, butyrate and
196 acetate were 2412 mg L^{-1}, 1470 mg L^{-1} and 2015 mg L^{-1}, respectively, in the end liq-
197 uid products. And the reducing sugar also did not accumulate to a detectable quantity
198 in the fermenting liquid.

199 3.1.5. Effects of L-cysteine concentration

200 Supplementation of reducing agent such as L-cysteine was an alternative way to
201 maintain the anaerobic environment. Moreover, L-cysteine as a mediator between
202 bacteria and substrate could reduce the oxidation-reduction potential (ORP) values of
203 the fermentation system and increase the growth rate of some bacteria.[38, 39] The results
204 of Yuan et al.[40] showed that L-cysteine could be used as a low-cost and highly effi-
205 cient bioactive agent to increase dark fermentative hydrogen production.

206 As shown in Fig. 5 (a) and (b), the hydrogen yield, cellulose degradation and end
207 liquid products increased with increase in L-cysteine concentration ranging from 0 to
208 0.7 g L^{-1}. The H_2 yield of *Clostridium acetobutylicum* X_9 + *Ethanoigenens harbinense*
209 B_2 and cellulose degradation reached a peak of 10.3 mmol g-MCC^{-1} and 85%, respec-
210 tively, at L-cysteine concentration of 0.7 g L^{-1}. Meanwhile, the maximum concentra-

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211 tions of ethanol, butyrate and acetate in the end liquid products were 2386 mg L^{-1},
212 1500 mg L^{-1} and 2032 mg L^{-1}, respectively. As shown in Fig.3 (c), the cell dry weight
213 increased with increase in L-cysteine concentration. Similarly, in the fermenting liq-
214 uid, the reducing sugar did not accumulate to a detectable quantity.

215 3.2. Orthogonal experiment

216 Based on single-factor experiments, the factors that influence the hydrogen yield were
217 examined through the orthogonal experiment. The design and results of the orthogo-
218 nal experiment $L_{16}(4^5)$ were presented in Table 1 and 2. The parameter K was the sta-
219 tistical average of hydrogen yield at one level (for one factor). The parameter R was
220 the statistical range of K_1-K_4 for one factor. The different values of K showed the ef-
221 fects of the four levels on hydrogen yield, while the different values of R suggested
222 the effects of the five factors on hydrogen yield.[41]

223 According to data analysis in Table 2, the order of influence strength was initial
224 pH > substrate concentration > incubation time > L-cysteine concentration > C/N. The
225 optimum hydrogen yield condition was initial pH 6.0，substrate concentration 12 g L^{-1}，
226 incubation time 40 h，L-cysteine concentration 0.7 g L^{-1} and C/N 4.0. Verification
227 experiment was carried out under the optimal condition, and the hydrogen yield was
228 10.4 mmol g-MCC^{-1}.

229 The stain B_2 and X_9 have different ability of bio-hydrogen production from cellu-
230 lose. In the mono-culture test, the X_9 achieved much higher hydrogen yield than B_2[42].
231 In the co-culture of B_2 and X_9 test, a maximum hydrogen yield of 10.4 mmol
232 g-MCC-1 was obtained under the optimum condition, which was approximately

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233 2.2-fold greater than the mono-culture *Clostridium acetobutylicum* X_9[40]. It reveals that
234 the co-culture of strain B_2 and X_9 can achieve bioaugmentation effects for hydrogen
235 production from cellulose.
236 Moreover, the efficiency of X_9+B_2 co-culture cellulosic H_2 production system is
237 comparable to that reported in the other studies (Table 3). The results indicated that
238 co-culture of Clostridium acetobutylicum X_9 + Ethanoigenens harbinense B_2 presents
239 a potential approach to converting cellulose into hydrogen energy. **4 Conclusions**
240 This study explored the optimization of key factors affecting biohydrogen production
241 from microcrystalline cellulose by co-culture of isolated strains *Clostridium acetobu-*
242 *tylicum* X_9 + *Ethanoigenens harbinense* B_2. In single-factor experiment, hydrogen
243 production, end liquid products, cellulose degradation, quantities of reductive saccha-
244 rides were measured versus initial pH, substrate concentration, incubation time, C/N
245 and L-cysteine concentration, respectively. Based on single-factor experiments, the
246 factors that influence the hydrogen yield were examined through the orthogonal ex-
247 periment. The sequence of influence strength of the factors was initial pH > substrate
248 concentration > incubation time > L-cysteine concentration > C/N. The determined
249 optimal conditions were initial pH 6.0, substrate concentration 12 g L^{-1}, incubation
250 time 40 h, L-cysteine concentration 0.7 g L^{-1} and C/N 4.0. Under the optimum con-
251 dition, a maximum hydrogen yield of 10.4 mmol g-MCC^{-1} was obtained, which was
252 approximately 2.2-fold greater than the mono-culture *Clostridium acetobutylicum* X_9
253 in our previous study.[42] The two strains were isolated from the same habitat.There
254 exists ecological niche complementarity between them. The corresponding end liquid

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255 products were acetate, ethanol, and butyrate. In stable hydrogen production phase,
256 ethanol content was 2400mg/L, which was 1.6-fold greater than butyrate. The
257 increasing neutral ethanol content and component could avoid effect of acid products
258 on microbial metabolic processes. In the stage pH range of variation, high cellulose
259 degradation, hydrogen production and microbes activity would be maintained, and
260 hydrogen production cycle would be prolonged. Hence, the co-culture of strain Etha-
261 noigenens harbinense B_2 and *Clostridium acetobutylicum* X_9 can achieve bioaugmen-
262 tation effects for hydrogen production from cellulose and is more competitive than the
263 mono-culture in cellulose conversion. Our research results indicated that dark fer-
264 mentation of cellulosic biomass by co-culture of *Clostridium acetobutylicum* X_9 +
265 Ethanoigenens harbinense B_2 should be further developed. It has potential use for
266 converting cellulose and hemicellulose into hydrogen energy.

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274 **References**

275 1. I. K. Kapdan and F. Kargi, *Enzyme. Microb. Tech.*, 2006, **38**, 569-582.
276 2. A. Midilli, M. Ay, I. Dincer and M. A. Rosen, *Renew. Sust. Energ. Rev.*, 2005, **9**,
277 255-271.
278 3. Z.y. Zhao and H. Yan, *Renew. Energ.*, 2012, **37**, 53-60.

RSC Advances Accepted Manuscript

279 4. J. Li and J. Ge, *Procedia Environmen.Sci.*, 2011, **10**, 2153-2158.
280 5. E. Kırtay, *Energ.Convers.Manage.*, 2011, **52**, 1778-1789.
281 6. K. Zhang, N. Ren, C. Guo, A. Wang and G. Cao, *J.Environ.Sci.*, 2011, **23**,
282 1929-1936.
283 7. F. Xu, K. Theerarattananoon, X. Wu, L. Pena, Y.C. Shi, S. Staggenborg and D.
284 Wang, *Ind. Crops Prod.*, 2011, **34**, 1212-1218.
285 8. M. Asgher, Z. Ahmad and H. M. N. Iqbal, *Ind. Crops Prod.*, 2013, **44**, 488-495.
286 9. Q. Zhang, L. Tang, J. Zhang, Z. Mao and L. Jiang, *Bioresour. Technol.*, 2011, **102**,
287 3958-3965.
288 10. X. Y. Cheng, Q. Li and C. Z. Liu, *Bioresour. Technol.*, 2012, **114**, 327-333.
289 11. D. Levin, *Int. J. Hydrogen Energ.*, 2004, **29**, 173-185.
290 12. Q. Li and C.-Z. Liu, *Int. J. Hydrogen Energ.*, 2012, **37**, 10648-10654.
291 13. Alibardi and Cossu, *Waste Management* , 2015,**36**, 147-155.
292 14. N. Ren, A. Wang, G. Cao, J. Xu and L. Gao, *Biotechnol. Adv.*, 2009, **27**,
293 1051-1060.
294 15. M. Cui, Z. Yuan, X. Zhi, L. Wei and J. Shen, *Int. J. Hydrogen Energ.*, 2010, **35**,
295 4041-4047.
296 16. R. Datar, J. Huang, P. Maness, A. Mohagheghi, S. Czernik and E. Chornet, *Int. J.*
297 *Hydrogen Energ.*, 2007, **32**, 932-939.
298 17. I. A. Panagiotopoulos, R. R. Bakker, T. de Vrije, E. G. Koukios and P. A. M.
299 Claassen, *Int. J. Hydrogen Energ.*, 2010, **35**, 7738-7747.
300 18. A. Ghimire, L. Frunzo, F. Pirozzi., E. Trably and R. Escudie. 2015, *Appl. Energ.*,
301 **144**, 73–95.
302 19. Favaro et al., Int. J. Hydrogen Energ. 2013, **38**, 11774-11779.
303 20. A. M. Adel, Z. H. Abd El-Wahab, A. A. Ibrahim and M. T. Al-Shemy, *Carbohyd.*
304 *Polym.*, 2011, **83**, 676-687.
305 21.A.P.Mathew, K.Oksman, M.Sain. *J. Appl Polym Sci*, 2005, **97(5)**,2014-2025.
306 22. A. Geng, Y. He, C. Qian, X. Yan and Z. Zhou, *Bioresour. Technol.*, 2010, **101**,
307 4029-4033.
308 23. C.H. Chou, C.-L. Han, J.-J. Chang and J.-J. Lay, *Int. J. Hydrogen Energy*, 2011,
309 **36**, 13972-13983.
310 24. S. Wu, X. Li, J. Yu and Q. Wang, *Bioresour. Technol.*, 2012, **123**, 184-188.
311 25. S. Ma, H. Wang, Y. Wang, H. Bu and J. Bai, *Renew. Energ.*, 2011, **36**, 709-713.
312 26. A. Wang, *Int. J. Hydrogen Energy*, 2008, **33**, 912-917.
313 27. N. Q. Ren, X. Q. Wang, W. S. Xiang, M. Lin, J. Z. Li and W. Q. Guo, *High.*
314 *Technol .Lett* .2002, **8**, 21-25.
315 28.H.Minato, A. Endo,H. Kouriyama,T.Uemura, Nippon Nogeikagaku Kaishi, 1962,
316 **36**, 101-106.
317 29. Miller, G.L., 1959.J.Anal.Chem-engl.Tr., *Analytical Chemistry*, **31**, 426–428.
318 30. Y. C. Lo, M. D. Bai, W. M. Chen and J. S. Chang, *Bioresour. Technol.*, 2008, **99**,
319 8299-8303.
320 31. Y. Fan, *Bioresour. Technol.*, 2004, **91**, 189-193.
321 32. N. Q. Ren, B. Z. Wang and J. C. Huang, *Biotechnol. Bioeng.*, 1997, **54**, 428-433.
322 33. S. V. Ginkel, S. Sung and J.-J. Lay, *Environ.Sci.Technol.*, 2001, **35**, 4726-4730.
323 34. C. Sreela-or, T. Imai, P. Plangklang and A. Reungsang, *Int. J. Hydrogen Energ.*,
324 2011, **36**, 14120-14133.
325 35. Y. C. Lo, Y. C. Su, C. Y. Chen, W. M. Chen, K. S. Lee and J. S. Chang,
326 *Bioresour. Technol.*, 2009, **100**, 5802-5807.
327 36. C. Lin, *Int. J. Hydrogen Energy*, 2004, **29**, 41-45.
328 37. Q. Li, D. Xing, N. Ren, L. Zhao and Y. Song, *J. Environ. Sci.*, 2006, **27**, 810-814.

RSC Advances Accepted Manuscript

329 38. R.a. Doong and B. Schink, *Environ. Sci. Technol.*, 2002, **36**, 2939-2945.
330 39. Y. Song and B. E. Logan, *Water Res.*, 2004, **38**, 1626-1632.
331 40. Z. Yuan, H. Yang, X. Zhi and J. Shen, *Int. J. Hydrogen Energ.*, 2008, **33**,
332 6535-6540.
333 41. H. Su, J. Cheng, J. Zhou, W. Song and K. Cen, *Int. J. Hydrogen Energ.*, 2009, **34**,
334 8846-8853.
335 42. H. X. Bao, W. W. Cai, X. P. Ma, Y. T. Song, M. L. Shen, Z. L. Chen, L. D. Li and
336 N. Q. Ren, Adv. Mater. Res., 2012, 512-515, 1446-1449.
337 43. A.Wang, N.Ren, Y.Shi, et al., Int. J. Hydrogen Energ., 2008, **33**, 912-917.
338 44. Y.Liu, P.Yu, X.Song, et al., *Int. J. Hydrogen Energ*., 2008, **33**, 2927-2933.
339 45. A.Wang,L. Gao, N.Ren, et al. *Biotechnol. Lett.*, 2009, **31**, 1321-1326.
340 46. Y.C.Lo, M.D.Bai, W.M.Chen, J.S.Chang, Bioresource Technol., 2008,
341 99,8299-8303.

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342 **Table 1** Results of $L_{16}(4^5)$ orthogonal design.

Experiment no.	Parameters					R_{H2} [c] (mmol g^{-1})
	SC [a] (g L^{-1})	Initial pH	C/N	Incubation time (h)	LC [b] (g L^{-1})	
1	10	5.0	2.5	36	0.3	8.5
2	10	5.5	3.3	38	0.5	9.6
3	10	6.0	4.0	40	0.7	10.1
4	10	6.5	5.0	42	1.0	9.7
5	12	5.0	3.3	40	1.0	9.1
6	12	5.5	2.5	42	0.7	9.9
7	12	6.0	5.0	38	0.5	10.3
8	12	6.5	4.0	36	0.3	9.4
9	15	5.0	4.0	42	0.5	8.7
10	15	5.5	5.0	40	0.3	9.7
11	15	6.0	2.5	38	1.0	9.8
12	15	6.5	3.3	36	0.7	9.2
13	18	5.0	5.0	38	0.7	8.3
14	18	5.5	4.0	36	1.0	8.6
15	18	6.0	3.3	42	0.3	9.0
16	18	6.5	2.5	40	0.5	9.3

343 [a] SC represents substrate concentration.

344 [b] LC represents L-cysteine concentration.

345 [c] R_{H2} represents hydrogen yield.

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Table 2 Analysis of $L_{16}(4^5)$ experiment results.

	Hydrogen yield				
	SC (g L^{-1})	**Initial pH**	**C/N**	**Incubation time (h)**	**LC (g L^{-1})**
K_1[a]	9.475	8.650	9.375	9.150	9.150
K_2	9.675	9.450	9.225	9.275	9.375
K_3	9.350	9.800	9.500	9.550	9.475
K_4	8.800	9.400	9.200	9.325	9.300
R[b]	0.875	1.150	0.300	0.400	0.325
Optimal result	SC_2	pH_3	C/N_3	$time_3$	LC_3

[a] K represents the average of hydrogen yield of four experiments at one level (for one factor).

[b] R represents the range of K_1-K_4 for one factor.

Table 3 Comparison of H_2 production performance using cellulosic material as substrate

Microbe	**Substrate**	**Temperature (℃)**	**H_2 yield (mmol/g)**	**Reference**
X9	Stream-exploded corn stover (15g/L)	37□	3.4	Wang et al.[43]
X9 + B49	MCC (10g/L)	38□	8.1	Wang et al.[43]
JN4 + GD17	MCC, filter paper or cellobiose (5g/L)	60□	18	Liu et al.[44]
G1 + B49	MCC(5g/L)	37□	2.97	Wang et al.[45]
Sludge & Clostridium pasteurianum	CMC(10g/L)	35□	1.09	Lo et al.[46]
X9 + B9	MCC(12g/L)	37□	10.4	This study

MC Microcrystalline cellulose, *CMC* Carboxymethyl cellulose

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356 **Figure captions**

357 **Fig. 1. (a)** Effects of initial pH on H_2 yield/Cellulose degradation; **(b)** Effect of initial
358 pH on End liquid products/Reduced Sugar of X_9+B_2; **(c)** Effects of pH on Cell dry
359 weight.

360 **Fig. 2. (a)** Effects of substrate concentration on H_2 yield/Cellulose degradation; **(b)**
361 Effects of substrate concentration on End liquid products/Reduced Sugar of X_9+B_2; **(c)**
362 Effects of substrate concentration on Cell dry weight.

363 **Fig. 3. (a)** Effects of incubation time on H_2 yield/Cellulose degradation; **(b)** Effects of
364 incubation time on End liquid products/Reduced Sugar of X_9+B_2; **(c)** Effects of
365 incubation time on Cell dry weight.

366 **Fig. 4. (a)** Effects of C/N on H_2 yield/Cellulose degradation; (b) Effects of C/N on
367 End liquid products/Reduced Sugar of X_9+B_2; **(c)** Effects of C/N on Cell dry weight.

368 **Fig. 5. (a)** Effects of L-cysteine concentration on H_2 yield/Cellulose degradation; **(b)**
369 Effects of L-cysteine concentration on End liquid products/Reduced Sugar of X_9+B_2;
370 **(c)** Effects of L-cysteine on Cell dry weight.

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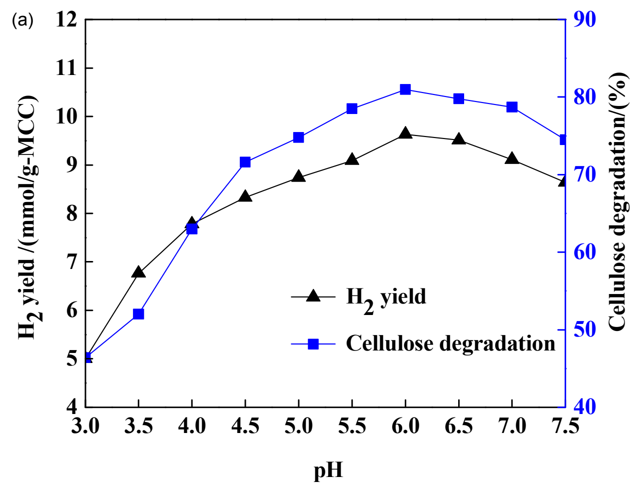
374 **Fig.1**



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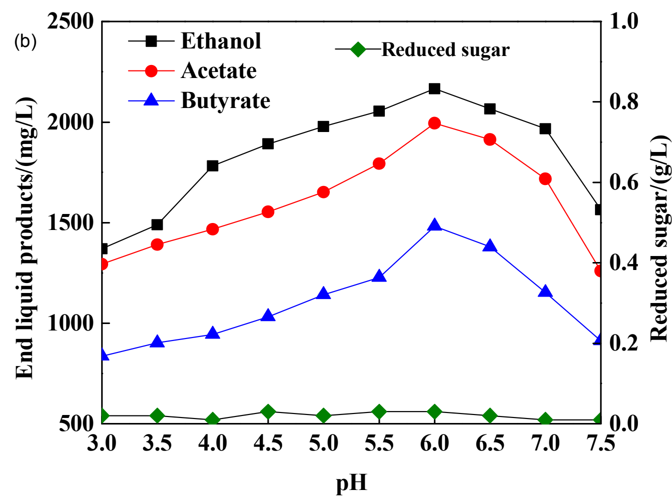

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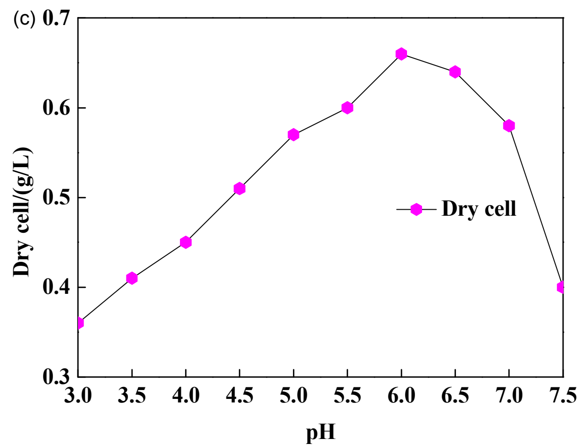

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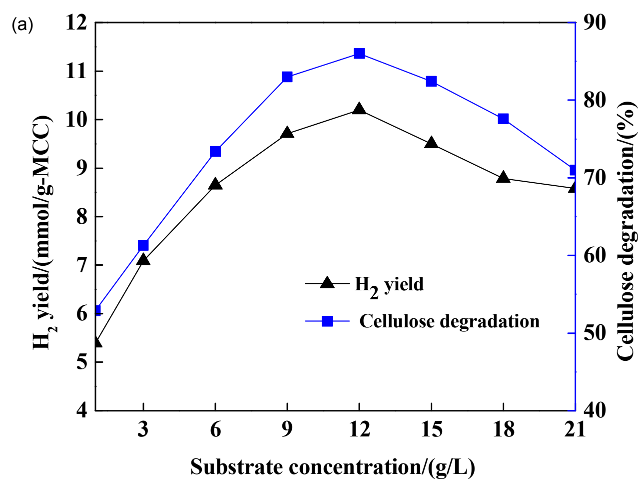
378 **Fig.2**



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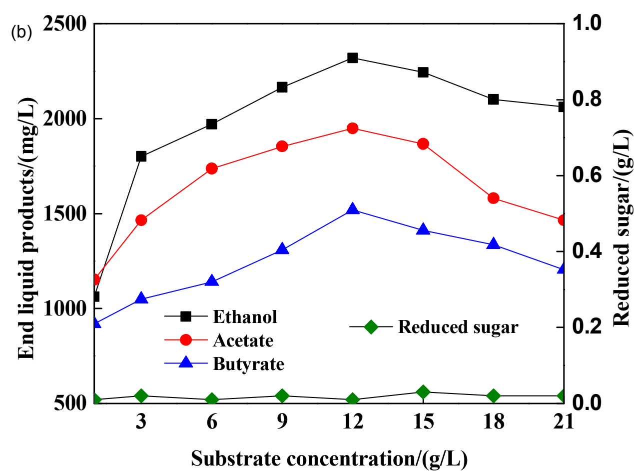

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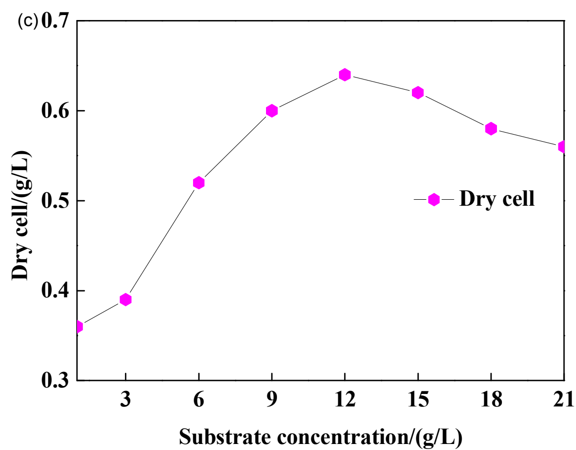

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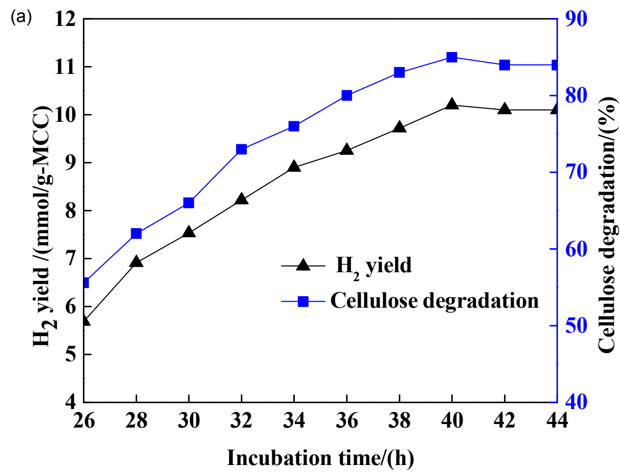
382 **Fig.3**



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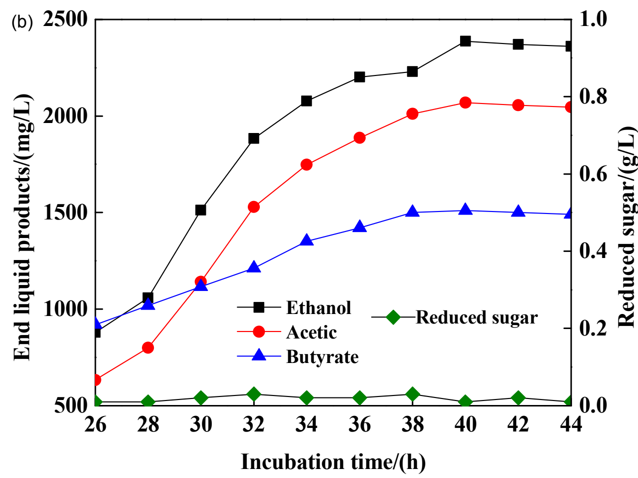

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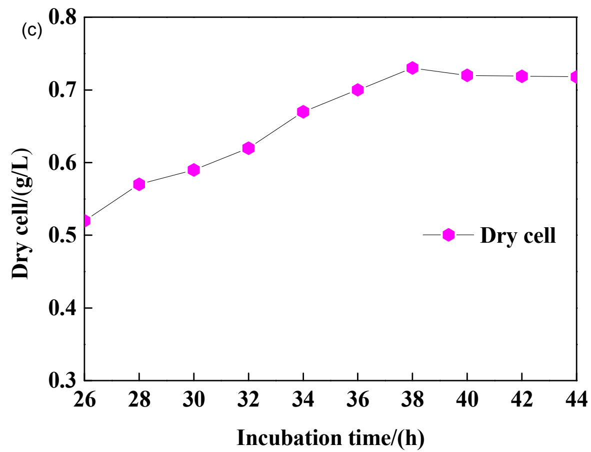

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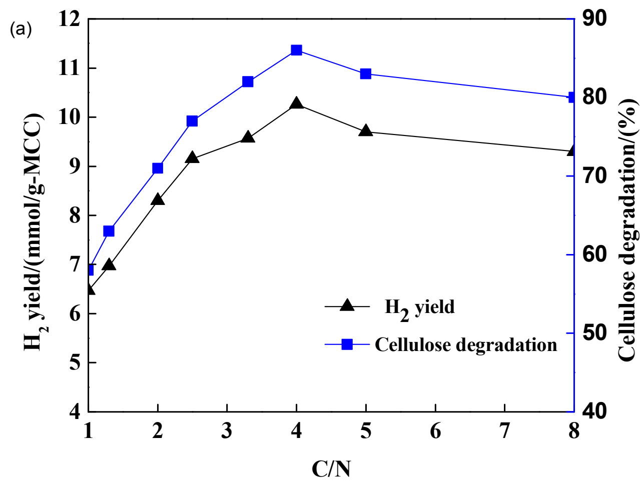
386 **Fig.4**



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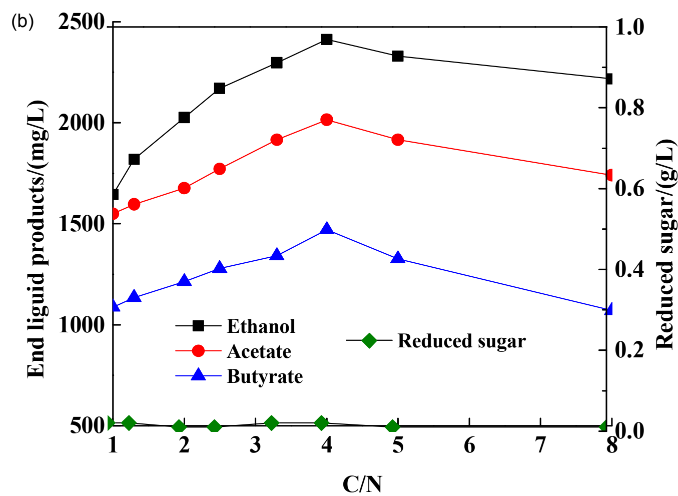

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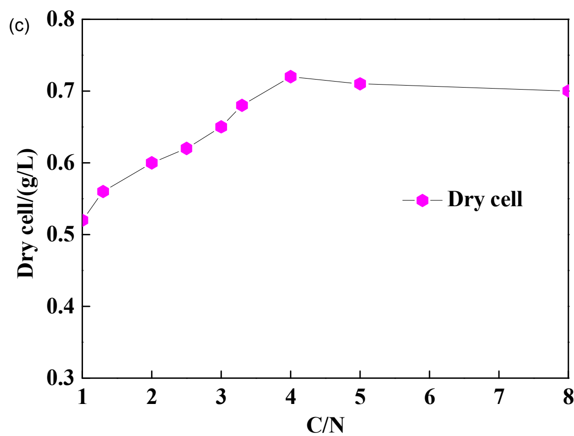

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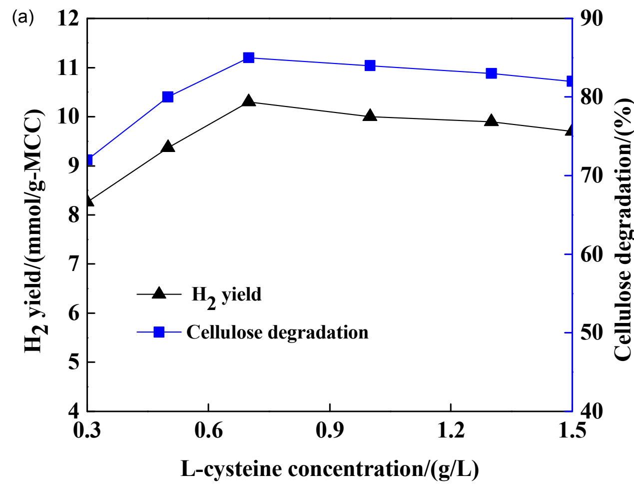
390 **Fig.5**



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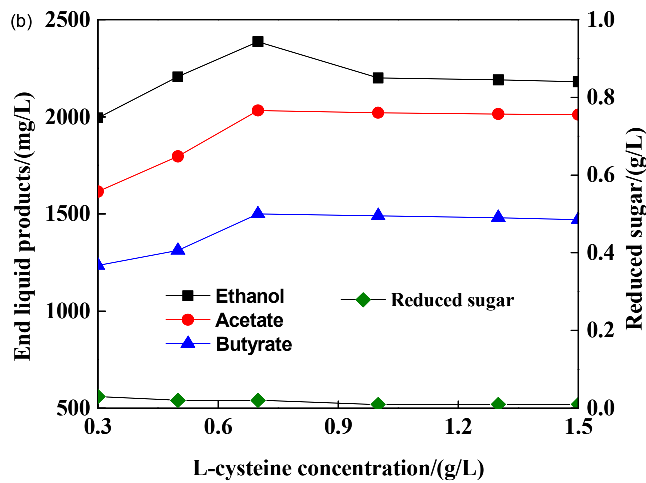

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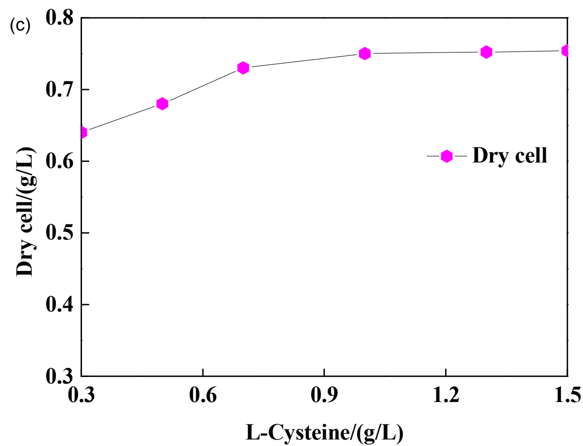

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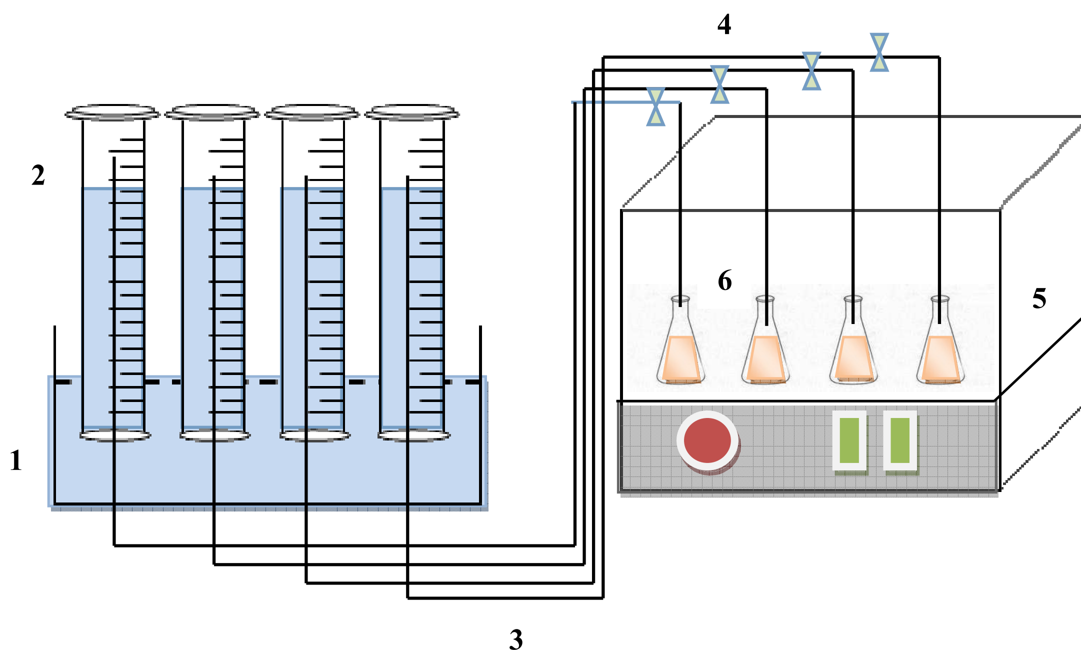

1.Sink; 2.Graduated cylinder; 3.Valve; 4.Catheter; 5.Air bath oscillator; 6.Serum bottle

The strains (X9+B2) were co-cultured in several serum bottles. Hydrogen was gathered by a series of graduated cylinders.

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