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## CO<sub>2</sub> valorization by a new microbiological process

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**Abstract.** Carbon dioxide constitutes one of the largest carbon feedstocks for fuel production. This work demonstrates that an environmental bacterial consortium grown on methane is able to directly and selectively reduce CO<sub>2</sub> into formate. The conversion was carried out at 30°C and atmospheric pressure, without any the addition of organic molecules (such as cofactor), photons or H<sub>2</sub> to the reaction medium. When exposed to a CO<sub>2</sub>:air (1:1 v/v) mixture, the consortium was able to produce  $280 \pm 10 \text{ mg} \cdot \text{g}_{\text{dry cell}}^{-1}$  of formate in 15 days. A control experiment performed solely with air coupled to NMR analysis suggested that at least 17 % of the formate was produced from direct CO<sub>2</sub> reduction. The formate produced could be used as a precursor for methanol production from methane by methanotrophic bacteria or it could be acidified to feed Direct Formic Acid Fuel Cells.

**Key words.** Carbon dioxide, reduction, formate, bio-catalytic process, microbial process.

## 25 1. Introduction

26 Carbon dioxide (CO<sub>2</sub>) accounts for more than 75% of global greenhouse gas (GHG) emissions, with annual  
27 anthropogenic emissions of about 25 to 35 Gt [1]. These emissions, which today represent around 65% of GHG  
28 emissions in CO<sub>2</sub> equivalents [1], derive mainly from heavy and energy producing industries (cement plants,  
29 aluminum and steel production sites, coal and oil-fired power plants), as well as transport [1, 2]. Use of CO<sub>2</sub> as  
30 a raw material at the industrial level is currently being studied as a possible solution to reduce levels of this gas  
31 in the atmosphere. However, today, industrially, CO<sub>2</sub> is mainly used as a fluid rather than a reactant and it  
32 originates mainly from natural sources or fermentation plants, its industrial use representing less than 1% (i.e.  
33 100 Mt/yr) of annual emissions [2]. Indeed, industrial use of the gas as a chemical reactant is currently very  
34 limited and based on the affinity of some nucleophilic molecules for CO<sub>2</sub> to form urea, carbamates and cyclic  
35 organic carbonates [3].

36 In this context, over the last fifty years direct conversion of anthropogenic CO<sub>2</sub> into alternative fuels (such as  
37 methane, C<sub>1</sub>-C<sub>5</sub> alcohols or formic acid) has become a major research topic because it could help solve two  
38 major future challenges, namely climate change and fossil fuel shortages. Nevertheless, activation of CO<sub>2</sub>  
39 molecules is very difficult because dissociation of the C=O bond requires a large amount of energy (i.e. 795  
40 kJ.mol<sup>-1</sup>). Activation of CO<sub>2</sub> therefore requires efficient and selective catalysts but also, quite often, relatively  
41 high temperatures and pressures.

42 Both homogeneous and heterogeneous catalysts have been reported to catalyze hydrogenation reactions [2, 4,  
43 5], artificial photosynthesis [6-10] and electrochemical reduction [3, 11, 12], giving rise to fuels and molecule  
44 platforms from CO<sub>2</sub>. However, despite recent progress, these chemical conversion routes still exhibit low  
45 durability and/or selectivity [6, 13-15].

46 The biological processes for CO<sub>2</sub> activation are interesting because they are usually selective and require mild  
47 conditions. These biological processes are considered sustainable as they are low-energy-consuming and  
48 reagent use and/or by-product release that could have a negative environmental impact is generally limited.  
49 Currently, only processes implementing photosynthetic microorganisms such as microalgae or cyanobacteria  
50 have been developed at the industrial scale. The main products recovered from CO<sub>2</sub> valorization by these  
51 processes are lipids which can be then converted into biodiesel [16, 17]. Open raceway technology has allowed  
52 the scaling-up of microalgae processes but their development is still limited due to (i) nutrients and mass transfer  
53 limitations, (ii) the need for good sunlight capture with special bioreactors requiring very large areas and low  
54 depths, (iii) the need to maintain a basic pH (8-10), and (iv) the need to harvest the microorganisms in order to  
55 extract the compounds of interest. All these constraints limit productivity and generate relatively high costs  
56 [18]. At the laboratory scale, cyanobacteria have been genetically modified to produce alcohols directly from  
57 CO<sub>2</sub> [18-21]. Very recently, the carbon assimilation pathway of the cyanobacterium *Synechococcus elongatus*  
58 PCC 7942 has been directed towards the production of 2,3-butanediol from CO<sub>2</sub> and glucose in the dark [22].  
59 Such an advance overcomes the limitations related to the need for sunlight, but the need to add glucose still  
60 makes the bioprocess unattractive from an economic point of view. In addition, the biological stability of the  
61 modified cyanobacteria has yet to be improved for industrialization purposes [18-22].

62 Among the biological catalysts, Formate Dehydrogenase (FDH) enzymes are known to be reversible and several  
63 works have focused on the selective reduction reaction of CO<sub>2</sub> into formate by extracted, purified and improved  
64 FDH [23, 24]. Recently, the enzyme nitrogenase, a well-known N<sub>2</sub>-reducing enzyme, has also been reported to  
65 be able to catalyze the conversion of CO<sub>2</sub> into formate and methane [25, 26]. However, the scaling-up of this  
66 bio-conversion process is difficult because it requires the addition of costly cofactors to provide the essential  
67 electrons and protons for CO<sub>2</sub> bio-reduction [27, 28]. Electrochemically assisted reactors (also known as bio-  
68 electrolyzers), inoculated with electro-active litho-autotrophic bacteria, constitute another biological means of  
69 CO<sub>2</sub> conversion [16, 29-34]. Two different configurations are possible with these systems: (1) CO<sub>2</sub> reduction  
70 may be catalyzed by the biotic cathode and the required electrons are supplied by the polarized cathode while  
71 the protons derive from water oxidation at the anode or (2), in the case of use of hydrogenotrophic bacteria, the  
72 cathode electrons and the protons arising from the anode can be used *in situ* to generate dihydrogen (H<sub>2</sub>) that  
73 will then be assimilated by the bacteria. Recently, a methylotrophic bacterium, *Methylobacterium extorquens*  
74 AM1, was reported to have produced formate from CO<sub>2</sub> with the first bio-electrolyzer configuration, but no  
75 cathodic current driven by the presence of CO<sub>2</sub> was evidenced and the CO<sub>2</sub> reduction reaction was not  
76 demonstrated [31]. When hydrogenotrophic bacteria were used with the second configuration, different  
77 products such as methane [31], acetate [32], PolyHydroxyButyrate (PHB), biomass and C3-C5 alcohols [33]  
78 were obtained from CO<sub>2</sub> and H<sub>2</sub>. However, the fact that hydrogen (H<sub>2</sub>) is an expensive energy vector and its  
79 solubility in water is low constitute major drawbacks for the scaling-up of this process that produces molecules  
80 with a lower energy potential than H<sub>2</sub>.

81 The hydrogenotrophic bacterium *Ralstonia eutropha* H16 has been genetically modified to use formate as a  
82 source of carbon and protons instead of CO<sub>2</sub> and H<sub>2</sub> to produce isobutanol and 1-methyl-butanol [34]. Bio-  
83 electrolyzer tests were carried out with the bacterium to try and generate formate *in situ* at the cathode by  
84 reduction of CO<sub>2</sub> [34], but electrochemical reduction of CO<sub>2</sub> still remains difficult.

85 Finally, methanotrophic bacteria (*Methylosinus trichosporium* IMV 3011 and *Methylosinus sporium*) have been  
86 reported to selectively convert CO<sub>2</sub> into methanol with their enzymes (including FDH first that can reduce CO<sub>2</sub>  
87 into formate) and their intracellular PHB stock being used as a source of electrons and protons [35-37]. Contrary  
88 to the other biocatalysts, with these bacteria, there is no need to add costly chemicals such as cofactors or H<sub>2</sub> to  
89 the reaction medium, or to provide the reaction system with light, or to genetically modify the metabolic  
90 pathway of the bacteria to recover products directly from CO<sub>2</sub>. Moreover, methanotrophic bacteria use methane  
91 (CH<sub>4</sub>) as a carbon and energy source to grow, meaning the global carbon balance of the CO<sub>2</sub> valorization  
92 bioprocess is positive as our city and agricultural wastes are a renewable source of methane. Methanotrophic  
93 bacteria are thus interesting biocatalysts for CO<sub>2</sub> reduction. However, the methanol production obtained was  
94 very low (3.6 μmol<sub>methanol</sub>·g<sub>dry cell</sub><sup>-1</sup>).

95  
96 In this work, an environmental bacterial consortium rich in methanotrophic bacteria was shown to directly and  
97 selectively catalyze the reduction of CO<sub>2</sub> into formate, a chemical which could be considered as a future  
98 energetic vector since its acidic form, i.e. formic acid, can be used as fuel in Direct Formic Acid Fuel Cells  
99 (DFAFCs) or as an H<sub>2</sub>-storage molecule. Formate could also be employed as an electron donor for oxidation of

100 CH<sub>4</sub> into methanol by methanotrophic bacteria [38, 39]. Significant amounts of formate were able to be  
101 produced (1.06 mmol<sub>formate</sub> · g<sub>dry cell</sub><sup>-1</sup>) without the addition of costly chemicals to the reaction medium or *in situ*  
102 generating compounds for CO<sub>2</sub> reduction. In addition, the native consortium was grown on methane, a  
103 renewable source of carbon. This new CO<sub>2</sub> valorization route appears very promising and a patent application  
104 was therefore submitted [40], justifying the composition of the bacterial consortium remaining confidential  
105 until publication of said patent.

106

## 107 **2. Materials and methods**

108

### 109 **2.1. Bacteria and culture conditions**

110 A bacterial consortium [40] containing methanotrophic bacteria, the composition of which remains confidential,  
111 was grown in a modified nitrate mineral salts (NMS) medium enriched with copper and iron. The bacteria were  
112 grown in sealed vials incubated at 30°C on a rotary shaker (Unimax 1010, Heidolph) operated at 160 rpm. The  
113 headspace of the vials was filled with a mixture of CH<sub>4</sub> and air (1:1 v/v) that was used as the source of carbon  
114 and energy; the gas volume was 3-fold that of the liquid. The NMS medium was composed of 1.06 g/L KH<sub>2</sub>PO<sub>4</sub>,  
115 4.34 g/L Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 1.7 g/L NaNO<sub>3</sub>, 0.34 g/L K<sub>2</sub>SO<sub>4</sub> and 0.074 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O; the pH of NMS  
116 medium was adjusted to 7.0 ± 0.1 with NaOH, 0.1 M or HCl, 0.1 M and the medium was then autoclaved.  
117 Mineral, copper and iron solutions were prepared independently and sterilized by filtration on a 0.2-µm acetate  
118 cellulose membrane (Sartorius) before being added to the NMS medium. The final concentrations of the mineral  
119 solutions were 0.57 mg/L ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.446 mg/L MnSO<sub>4</sub>·H<sub>2</sub>O, 0.124 mg/L H<sub>3</sub>BO<sub>3</sub>, 0.096 mg/L  
120 Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.096 mg/L KI, 7.00 mg/L CaCl<sub>2</sub>·2H<sub>2</sub>O, that of the copper solution 0.798 mg/L CuSO<sub>4</sub>, and  
121 that of the iron solution 11.20 mg/L FeSO<sub>4</sub>·7H<sub>2</sub>O.

122

### 123 **2.2. Preparation of the bacterial suspension**

124 At the beginning of the stationary phase, the cultured cells were harvested by centrifugation at 4000 g for 20  
125 min at 4°C and then washed once with phosphate buffer 20 mM at pH 7.0. A reaction medium containing 20  
126 mM of phosphate buffer at pH 7.0 and 5 mM of MgCl<sub>2</sub> was used to re-suspend the cells to reach a final optical  
127 density at 600 nm (OD<sub>600</sub>) of about 10. Part of the resulting suspension was stored at -20°C with glycerol (20  
128 % w/w) for further biochemical analysis.

129

### 130 **2.3. CO<sub>2</sub>-reduction tests in batch mode**

131 The batch reactors were 60 mL-glass vials sealed with aluminium caps. The bacterial suspension was distributed  
132 into the batch reactors, with 6 mL being added per reactor. To assess the ability of the consortium to reduce  
133 CO<sub>2</sub>, several experiments were conducted with different headspace atmospheres (Table 1) that were first  
134 sterilized by filtration on 0.2-µm PTFE filters (Sartorius). For experiments A, B and C, a large set of vials was  
135 filled with the same bacterial suspension and incubated at 30°C under constant rotation (160 rpm). Each  
136 experiment was monitored for 20 days and at each sampling point, a vial was taken for analytical measurements.  
137 Different measurements were performed: optical density at 600 nm (OD<sub>600</sub>), pH, salts analysis by ionic

138 chromatography (IC) and quantification of the volatile organic compounds (VOC) contained in the liquid  
139 samples by headspace GC-MS.

140 Each experiment was conducted in triplicate. CO<sub>2</sub>-reduction tests were also carried out with <sup>13</sup>CO<sub>2</sub> to confirm  
141 the origin of the products arising from CO<sub>2</sub> reduction by carbon NMR analyses.

142

143 Table 1. Experimental conditions for the CO<sub>2</sub> reduction tests

144

Experiment	Presence of bacteria	Headspace atmosphere
A	Yes	CO <sub>2</sub> :Air (1:1 v/v)
B	Yes	Only Air (100 %)
C	Yes	Only N <sub>2</sub> (100%)
D	No	CO <sub>2</sub> :Air (1:1 v/v)

145

146 Air was taken from the atmosphere while CO<sub>2</sub> (99.995 %) and N<sub>2</sub> (99.995 %) were obtained from Air Liquide  
147 and <sup>13</sup>CO<sub>2</sub> (99.9 %) was obtained from Sigma Aldrich.

148

#### 149 **2.4. Sample processing**

150 At various reaction times, the bacterial suspensions in the reaction vials (i.e. 6 mL) were recovered and divided  
151 into different fractions. A first fraction was used to measure pH and OD<sub>600</sub>. A second fraction was centrifuged  
152 at 10 000 *g* at 4°C for 10 min; the supernatant was then recovered to be immediately analyzed (by GC-MS or  
153 ionic chromatography). In case of NMR characterization, a portion of the freshly sampled suspension (0.5 mL)  
154 was transferred into NMR tubes for direct analysis.

155

#### 156 **2.5. Analytical methods**

157 Optical density (OD<sub>600</sub>) was measured at 600 nm with a UV-2400 spectrophotometer (Shimadzu, Japan) and  
158 pH was measured with a C831 pH-meter (Consort, Belgium).

159 Volatile organic compounds, including methanol, ethanol, formaldehyde and acetaldehyde, were quantified by  
160 gas chromatography (Clarus 580, Perkin Elmer), coupled to mass spectrometry (Clarus SQ-8-MS, Perkin  
161 Elmer). A headspace system (Turbomatrix 16S, Perkin Elmer) was used for sample injection. Prior to analysis,  
162 acetonitrile was used as an internal standard at a final concentration of 39.3 ± 0.2 mg·L<sup>-1</sup>. The method included  
163 a 15-min sample heating step to 75°C followed by injection into the GC system. Separation was carried out  
164 using a capillary column (Rt-Q-Bond Plot, Restek) under isothermal conditions (150°C for 25 min). Calibration  
165 curves were obtained independently for each VOC.

166 Salt quantification of supernatants was carried out by ionic chromatography (IC). Sample portions of 25 µL  
167 were injected into a Dionex ICS-1000 system (Thermo Scientific™) with an IonPac AS19 (0.4 x 250 mm)  
168 capillary column and elution was carried out under the following conditions: 10 mM KOH (0 to 10 min), then  
169 10-45 mM KOH (10 to 30 min).

170 For NMR analysis, 450  $\mu\text{L}$  of the sample (complete suspension) was poured into a 5 mm NMR tube with 50  
 171  $\mu\text{L}$  of  $\text{D}_2\text{O}$ . The analyses were performed using a NMR BRUKER Avance III – 500MHz spectrometer –  
 172 CryoProbe Helium system for four hours. Different standards prepared in the reaction medium were analyzed  
 173 independently by NMR to obtain the carbon spectra of these molecules.

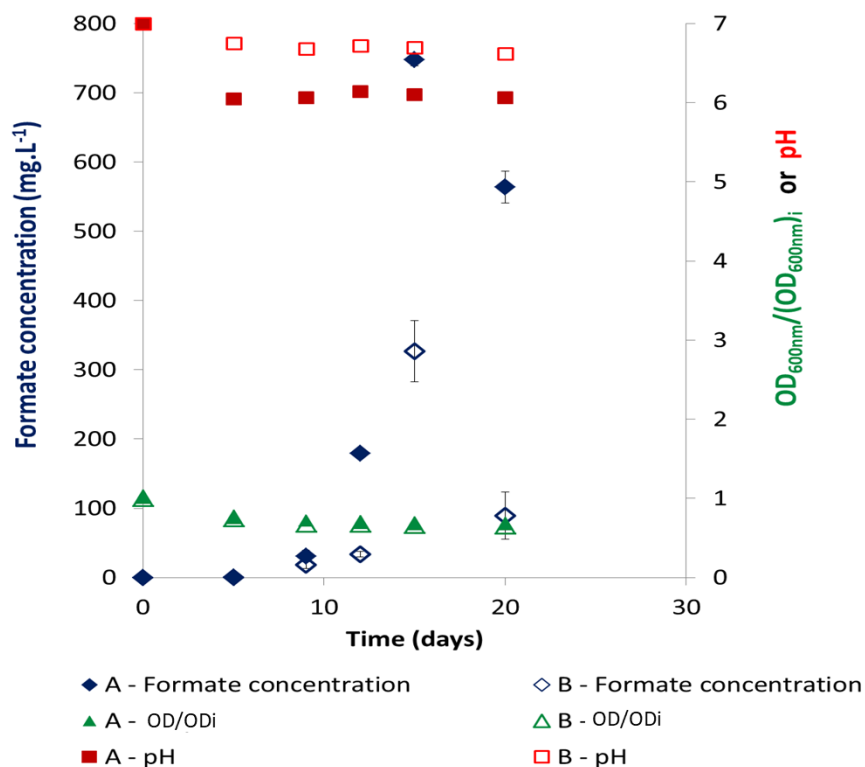
174

### 175 3. Results and Discussion

176 As detailed in Table 1, different experimental conditions were used to check the ability of the consortium to  
 177 reduce  $\text{CO}_2$  into formate. The conditions of experiment A correspond to the reaction conditions for formate  
 178 production, i.e. bacterial consortium in presence of a  $\text{CO}_2$ :air mixture (1:1 v/v). Experiments B, C and D,  
 179 performed without adding  $\text{CO}_2$  (B, C) or without bacteria (D), were blanks or control tests. Experiments B and  
 180 C aimed to assess whether there was any possible microbial by-product release when the bacteria were placed  
 181 in presence of air (B) or  $\text{N}_2$  (C). Experiment D was performed without bacteria and in presence of  $\text{CO}_2$  to check  
 182 that spontaneous  $\text{CO}_2$  reduction could not occur.

183

184 Figure 1 presents the results obtained in the batch reactors where formate appeared in the reaction medium, i.e.  
 185 experiments A and B (Table 1); for each experiment, three independent runs were performed in parallel and  
 186 each point represents the mean values obtained.



187

188 **Figure 1.** Reaction profiles of formate production, pH and  $\text{OD}_{600}/(\text{OD}_{600})_i$  obtained for experiments A and  
 189 B. A: bacterial consortium with an equimolar  $\text{CO}_2$ :air mixture and B: bacterial consortium with only air;  
 190  $(\text{OD}_{600})_i$  means initial  $\text{OD}_{600}$ ;  $(\text{OD}_{600})_i = 7.6 \pm 0.6$

191 corresponding to 2.45 g dry cell.L-1.

192

193 Regarding OD<sub>600</sub>, a nearly 25 % decrease in bacterial concentration was observed during the first few days  
194 which then remained constant (from day 5), with no differences being observed between headspace atmospheres  
195 A and B (Fig. 1). As the initial OD<sub>600</sub> was high ( $7.6 \pm 0.6$ ), the biomass concentration still remained significant  
196 during the experiments despite the decrease. The observations regarding OD<sub>600</sub> were similar for experiment C  
197 (data not shown). The fact that methanotrophic bacteria have been reported to be very resistant and able to form  
198 spores in stressing conditions [41] could also explain the preservation of OD<sub>600</sub> over 20 days despite the fact  
199 that the bacteria were exposed to unusual conditions (i.e. absence of their substrate CH<sub>4</sub> and high CO<sub>2</sub> content).  
200 In the reactors where CO<sub>2</sub> was added (experiment A), pH decreased from 7.0 to  $6.4 \pm 0.2$  within 5 days and  
201 then remained at that value which corresponds to the first acid dissociation constant (pKa) of CO<sub>2</sub> in water. A  
202 similar change in pH was observed in the reactors of experiment D as they also contained high levels of CO<sub>2</sub>  
203 (data not shown). In the vials containing only air (experiment B), pH decreased slightly to  $6.8 \pm 0.1$  due to the  
204 presence of traces of CO<sub>2</sub> (i.e. 400 ppmv). When the headspace contained N<sub>2</sub> (experiment C), pH remained  
205 constant at its initial value, i.e.  $7.0 \pm 0.1$  (data not shown).

206

207 The control tests carried out with the consortium and N<sub>2</sub> (experiment C) showed no evidence of microbial by-  
208 product release or formation by GC-MS or ionic chromatography throughout the duration of the experiment.  
209 No products were detected either in the control tests performed without bacteria but with an equimolar mixture  
210 of CO<sub>2</sub> and air (experiment D), which confirmed that spontaneous CO<sub>2</sub> reduction did not occur.

211

212 In experiments A and B (consortium exposed to an equimolar mixture of CO<sub>2</sub> and air or to air), no VOC were  
213 detected by GC-MS. Nevertheless, ionic chromatography analyses demonstrated the appearance of formate  
214 after 8 days in both of the experiments (Fig. 1). The consortium was able to produce up to  $750 \text{ mg} \cdot \text{L}^{-1}$  of formate  
215 in the CO<sub>2</sub>:air mixture (1:1) at day 15 of the reaction, corresponding to a formate production of  $280 \pm 10 \text{ mg} \cdot \text{g}_{\text{dry-cell}}^{-1}$   
216 (taking into account the bacterial mass initially introduced into the reactors).

217

218 When the consortium was exposed to air (experiment B), which contains a small fraction of CO<sub>2</sub> (400 ppmv),  
219 formate production was lower than that of experiment A (Fig. 1). In fact,  $330 \text{ mg} \cdot \text{L}^{-1}$  of formate were produced  
220 corresponding to a production of  $145 \pm 15 \text{ mg} \cdot \text{g}_{\text{dry-cell}}^{-1}$ . These results show that formate production is CO<sub>2</sub>- and  
221 bacteria-dependant and suggest that formate could be formed from CO<sub>2</sub> present in the atmosphere.

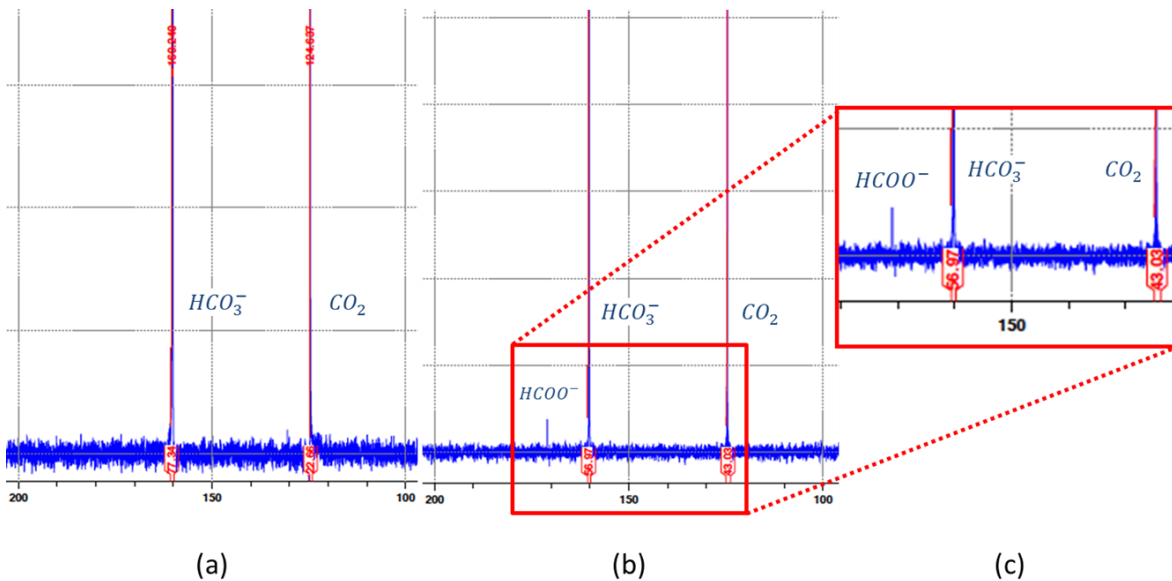
222 Nevertheless, the amount of formate obtained (i.e.  $330 \text{ mg} \cdot \text{L}^{-1} \times 6 \cdot 10^{-3} \text{ mL} = 1.98 \text{ mg}$ ) in experiment B (only  
223 air) was much higher than the expected theoretical quantity taking into account the CO<sub>2</sub> content of air (54 mL  
224 of air with 400 ppmv of CO<sub>2</sub> that can be transformed to a maximal formate mass of 0.04 mg). A possible  
225 explanation for the appearance of formate in excess in the reaction medium could be mixed acid fermentation.  
226 This bacterial phenomenon occurs under oxygen limitation and could give rise to products such as acetate and  
227 formate by cleavage of intracellular pyruvate by pyruvate formate lyase [42]. In this work, anoxic conditions



228 could have occurred due to a rapid depletion of the oxygen initially present in the reactors since oxygen is the  
229 usual final electron acceptor for the consortium; the bacterial consortium is indeed first grown under aerobic  
230 conditions before being exposed to CO<sub>2</sub> (section 2.1).

231

232 In order to confirm the actual ability of the consortium to reduce CO<sub>2</sub> into formate, a new set of experiments  
233 was carried out using a mixture of <sup>13</sup>CO<sub>2</sub>:air (1:1). The reaction was then monitored by <sup>13</sup>C NMR. Nuclear  
234 magnetic resonance spectroscopy (NMR) is only sensitive to <sup>13</sup>C since it has a spin number of ½. <sup>13</sup>CO<sub>2</sub>-  
235 labelling was consequently used to monitor any potential <sup>13</sup>C-containing product. In addition, the technique can  
236 be used to detect products irrespective of their localization (i.e. inside or outside the cells) as the NMR magnetic  
237 field can penetrate bacterial cells. The NMR spectrum obtained after 8 days of reaction is shown in Figure 2.



238

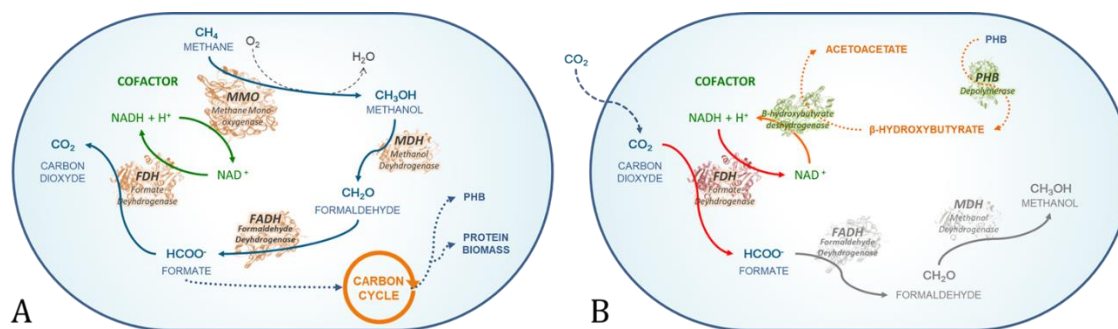
239 **Figure 2.** NMR spectrum at (a)  $t = 0$  days, where CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> are presents; (b)  $t = 8$  days showing a new  
240 compound with a chemical shift corresponding to formate, which is enlarged (c).

241

242 Resonance peaks for <sup>13</sup>CO<sub>2</sub> ( $\delta = 124.6$  ppm) and its corresponding ionized form H<sup>13</sup>CO<sub>3</sub><sup>-</sup> ( $\delta = 160.2$  ppm) were  
243 visible at the beginning. After 8 days of reaction, a new peak appeared ( $\delta = 170.9$  ppm), corresponding to the  
244 standard fingerprint of <sup>13</sup>C-labelled sodium formate. Since no other labelled compound was detected in the  
245 reaction medium, the labelled formate is thus likely to result from direct <sup>13</sup>CO<sub>2</sub> reduction. This result  
246 demonstrates that the consortium is thus able to reduce CO<sub>2</sub> into formate. However, in this experiment, it was  
247 not possible to recover semi-quantitative information about the labelled formate concentration produced since  
248 the <sup>13</sup>CO<sub>2</sub> and H<sup>13</sup>CO<sub>3</sub><sup>-</sup> signals were saturated. The only available information was that the formate  
249 concentration on day 8 was higher than the low NMR threshold for detection of labelled formate (i.e. 6 mg·L<sup>-1</sup>  
250 <sup>1</sup>). If it is considered that bacteria exposed to CO<sub>2</sub>:air (Fig. 1, run A) were able to produce 35 ± 2 mg·L<sup>-1</sup> of  
251 formate on day 9, it can be assumed that at least 17 % (= 6 / 35 x 100 %) of the formate produced resulted from  
252 CO<sub>2</sub> reduction.

253

254 Reduction of CO<sub>2</sub> by the consortium may be explained by several possible enzymatic cycles inside the bacteria.  
 255 Among the bacteria of the consortium, methanotrophic bacteria are supposed to be of CO<sub>2</sub> reduction catalysts.  
 256 These bacteria use CH<sub>4</sub> as an energy and carbon source. As described in Figure 3.A, the first step involves  
 257 methane oxidation to methanol by methanol monoxygenase (MMO); methanol is then successively converted  
 258 into formaldehyde and then formate by methanol dehydrogenase (MDH) and formaldehyde dehydrogenase  
 259 (FADH), respectively. Finally, formate dehydrogenase (FDH) catalyzes the oxidation of formate to CO<sub>2</sub>. The  
 260 methane consumed is fixed in the carbon cycle for protein and biomass production, and the CO<sub>2</sub> produced from  
 261 CH<sub>4</sub> oxidation is reported to be also mainly fixed in the carbon cycle through carboxylase enzymes  
 262 incorporating CO<sub>2</sub> into organic molecules already present inside the cells [43]. In addition, formate can also  
 263 enter the carbon cycle for the production of bacterial material, and PolyHydroxyButyrate (PHB) in particular.  
 264 PolyHydroxyButyrate is an energy storage polymer accumulated during the culture phase under oxygen and  
 265 nitrogen limitation [44, 45].  
 266



267  
 268 **Figure 3.** Methanotrophic bacteria: (A) pathway for CH<sub>4</sub> assimilation and (B) suggested pathway for CO<sub>2</sub>  
 269 reduction into formate.  
 270

271 A potential route for CO<sub>2</sub> assimilation by the bacteria is described in Figure 3.B. The CO<sub>2</sub> could indeed be  
 272 reduced into formate by the direct action of FDH and in that case, the equation balance of the reaction would  
 273 be:



275  
 276 where NADH is the reduced form of the Nicotinamide Adenine Dinucleotide (NAD) cofactor present in living  
 277 cells. However, the stock of endogenous NAD is finite. For the CO<sub>2</sub> reduction reaction to occur (Eq. 1), the  
 278 oxidized form of NAD (i.e. NAD<sup>+</sup>) has to also be continuously reduced into NADH (Fig. 3.B). Some  
 279 intracellular enzymes such as β-hydroxybutyrate dehydrogenase are able to recycle NAD<sup>+</sup> into NADH by  
 280 simultaneously oxidizing an electron and proton donor source (Fig. 3.B).  
 281

282 Compared to the work of Xin (2007) [36], no methanol traces were evidenced in the samples even if the  
 283 compound could have been potentially present in the reaction medium as a product of successive formate  
 284 reductions catalyzed by FADH and then MDH (Fig. 3.B). One possible explanation for this phenomenon could  
 285 be that formate production was so high that it led to inhibition of FADH.

286

287 Furthermore, formate was assayed in the supernatant of the samples, meaning that the formate was external to  
288 the cells. At this stage, it was assumed that the formate was produced inside the cells by the enzymatic  
289 machinery required for both CO<sub>2</sub> and NAD<sup>+</sup> reductions and then released out of the cells. The probability that  
290 these reactions were catalyzed by free enzymes resulting from cell lysis is low since the meeting probability  
291 between FDH, CO<sub>2</sub>, NAD, and the enzyme regenerating NAD is low. Regarding formate excretion from the  
292 cells, two phenomena could be assumed: (1) formate release could be due to a bacterial regulation process when  
293 intracellular formate concentrations become too high for cell viability as formate does indeed have antimicrobial  
294 properties [46] and its intracellular accumulation could lead to cell death, and (2) population differentiation  
295 could occur among the bacteria meaning that some bacteria could play the role of formate factories for the  
296 survival of other bacteria consuming the formate. Formate is effectively reported in the literature as a precursor  
297 used by methanotrophic bacteria for cell maintenance reactions [47, 48].

298

299 A 5-day period seems to correspond to the adaptation phase for bacteria and their enzymes to their new carbon  
300 substrate, i.e. CO<sub>2</sub> instead of CH<sub>4</sub>, before formate production occurs. A decrease in formate concentration (by  
301  $27 \pm 1\%$ ) was then observed after 15 days (Fig. 1). This could be due to formate consumption. Bacterial  
302 regulation or changes in bacterial population interactions or substrate limitation could be at the origin of this  
303 decrease. However, the theoretical amount of CO<sub>2</sub> available in the initial CO<sub>2</sub>:air mixture of the headspace was  
304 calculated according to the gas volume inside the vial (i.e. 54 mL) and the ideal gas equation. The calculated  
305 CO<sub>2</sub> available (i.e. 1.09 mmol) was nearly 10 times higher than the maximum theoretical amount of CO<sub>2</sub>  
306 consumed for formate production (i.e. 0.10 mmol) assessed using Eq. (1). This means that there was no CO<sub>2</sub>  
307 limitation and that the bacteria had an unlimited capacity for exogenous formate production; the result therefore  
308 indicates that the limiting factor was rather the source of electrons and protons (i.e. the internal PHB) required  
309 for NAD recycling and thus for CO<sub>2</sub> reduction into formate (Fig. 3.B).

310 The initial PHB dry mass content of the bacterial suspension was found to be about  $15 \pm 5\%$  (data not shown).  
311 A theoretical calculation, considering that PHB was composed of 1 000 monomer units, showed that a PHB  
312 mass content of approximately 10% would indeed make it possible to reach the maximum concentration of  
313 formate produced (i.e. 750 mg·L<sup>-1</sup>). However, the exact number of units making up intracellular PHB is  
314 unknown and PHB could also be used in other pathways such as mixed acid fermentation. This hypothesis  
315 therefore needs further investigation. Much more experimental work is also required to clarify the explanations  
316 provided above and to specify the metabolic ways of this interesting CO<sub>2</sub> activation bioprocess.

317

#### 318 **4. Conclusions**

319 A bacterial consortium containing methanotrophs was able to reduce CO<sub>2</sub> into formate with a positive carbon  
320 balance as bacterial biocatalysts were grown on methane, which is also a greenhouse gas. This bioprocess thus  
321 constitutes a new promising way to valorize CO<sub>2</sub>. The role of each bacterium composing the consortium will  
322 be further investigated to both fully understand the mechanism and optimize formate production from CO<sub>2</sub>. In

323 addition, formate production was shown to be enhanced by another phenomenon that is likely to be mixed acid  
324 fermentation. At this stage, it is assumed that 17% of the total formate produced resulted from CO<sub>2</sub> reduction;  
325 however, this content needs to be precisely quantified. The impact of operating conditions should also be studied  
326 in the future to control the contribution of CO<sub>2</sub> reduction on mixed acid fermentation. Finally, another way to  
327 optimize the process will focus on continuous formate recovery from the reaction medium and bipolar  
328 electro dialysis will also be tested.

329

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## 334 **6. References**

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