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Title	A First Marine <i>Vibrio</i> Biocatalyst to Produce Ethanol from Alginate, which is a Rich Polysaccharide in Brown Macroalgal Biomass
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2 **Title**

3 A First Marine *Vibrio* Biocatalyst to Produce Ethanol from Alginate, which is A Rich
4 Polysaccharide in Brown Macroalgal Biomass

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14

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19 The authors declare no competing interests.

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30 to the article and approved the submitted version.

31

32 **Running title**

33 A First *Vibrio* Biocatalyst to Produce Ethanol from Alginate

34

35 **Abstract**

36 The use of un-utilized feedstock and seawater for material and/or energy production using marine
37 microbial catalysts is one potential option towards contributing to the development of a more
38 sustainable society. Ethanol production from alginate, which is an oxidized polysaccharide present in
39 brown seaweed, is extremely difficult due to the imbalance of reducing power in the microbial cells.
40 Production of ethanol by such means has so far been unsuccessful using marine microbial biocatalysts.
41 To produce ethanol from alginate, an alternative pathway consisting of a pyruvate decarboxylase gene
42 (*pdc*) and an alcohol dehydrogenase II gene (*adhII*) derived from *Zymomonas mobilis* strain ZM4
43 was implemented into a metabolically engineered bacterium, *Vibrio halioticoli*, which is a
44 representative marine alginate decomposer. No ethanol from alginate was produced in the wild-type
45 *V. halioticoli*, however, the engineered *V. halioticoli* harboring the *pdc* and *adhII* operon (Pet operon),
46 designated to the *V. halioticoli* (Pet), was able to produce 880 mg/L ethanol in maximum from 1.5%
47 alginate for 72 h. The Pet operon also worked on the other marine alginolytic vibrios for ethanol
48 production from alginate. This is the first case of ethanol production from alginate using marine
49 bacterial biocatalysts under seawater-based media.

50

51 **Keywords:** vibrio, ethanol, metabolic engineering, seaweed, marine

52

53 INTRODUCTION

54 Sustainable energy production and supply are two of the critical global issues in developing
55 a more sustainable society. To combat the negative effects on both the environment and human society
56 due to the use of fossil fuels, the development of alternative renewable energy has been undertaken
57 since the late 1970's after the so-called 'oil shock' era [1-3]. In particular, the last four decades could
58 be defined as the era of biomass energy revolution; there have been a number of technical innovations
59 in the production of ethanol, hydrogen and methane using biomass as feedstock [1-2,4-7]. Among
60 these, bioethanol is the only fuel which is commercially available as a realistic gasoline
61 (transportation fuel) alternative. Generally, biomass energy uses land-based biomass, such as corn,
62 sugar cane and switch grass, as types of feedstock. This supply of biomass has caused a controversy
63 in the food vs energy debate (FvE) [e.g. 8,9]. To overcome these limitations, there have been many
64 attempts to use marine biomass as a feedstock for bioethanol production [8-15] because marine
65 biomass has many advantages not only in solving the FvE issue but also in its rapid growth, lack of
66 lignin, fewer fertilization uses, and soft texture capable of being milled and crushed by simple
67 mechanical operations [11-12,15]. The uses of marine biomass also could contribute to balancing CO₂
68 in the ocean to sustain Blue Carbon [16]. Traditional aquaculture has been capable of strengthening
69 regional seaweed production in Japan, Chile, China, Indonesia, the Philippines, South Korea, however,
70 bioethanol production from marine biomass from alginate is extremely difficult due to the absence of
71 traditionally matured biotechnologies in alcohol fermentation or in energy production using seaweed
72 as feedstock [9-12,15].

73 Alginate is a major component of brown algae showing viscous features [10]. The structure
74 is constructed by linear block copolymers of uronic acids; β -D-mannuronate (M) and α -L-guluronate
75 (G). They are arranged in homopolymeric and heteropolymeric forms; poly-mannuronate (polyM)
76 and poly-guluronate (polyG) are for homo- and MG random, respectively [15,17]. The high oxidative
77 state of the polyuronate structure also forces the use of the Entner-Doudoroff pathway following the
78 TCA cycle in bacteria [10,12,18], which causes lower levels of alcohol production due to reducing

power and energy imbalances. Nevertheless, there have also been many reports on alginate-degrading microorganisms isolated from terrestrial and marine environments [19-21], however, up to now it has not been reported that wild-type strains of alginate assimilating bacteria could produce ethanol. In 2011, successful ethanol production from alginate by genetically engineered *Sphingomonas* sp. A1 was reported [10], and also by *E. coli* [12]. The *Sphingomonas* sp. A1 implemented Pet-operon and deleted *ldh* gene can ingest “intact” alginate through the super-channel and produced ethanol at a maximum 13 g/L from 5% (w/v) alginate as a substrate after 3-days culture under aerobic conditions [10]. Genetically engineered *E. coli* implemented with genes encoding alginate degradation, transport, and metabolism derived from *V. splendidus* and the production of ethanol (Pet)-operon produced 20 g/L ethanol from an artificially prepared substrate mixture consisting of alginate, mannitol, and glucose at a ratio of 5:8:1, which is determined in a wild seaweed, *Sacharina japonica* [12]. However, during the culture with seaweed, salt from both raw and dried seaweed caused lower cell growth and ethanol production in these bacteria from terrestrial origins because these bacteria show a low tolerance to salinity. Therefore, a better microbial biocatalyst more tolerant to salinity is needed rather than terrestrial bacteria in order to achieve efficient ethanol production from marine biomass.

Vibrio halitotocoli is a facultatively anaerobic, non-motile, alginolytic marine bacterium, isolated from *Haliotis discus hannai*, and produces ethanol from mannitol [19]. Nine more related species have been described, and they form a monophyletic clade, Halitotocoli [22-23]. The fermentation pathway was predicted to be similar to that of *E. coli* on the basis of genome-wide metabolic pathway reconstruction [23-24]. Ethanol production from alginate has never been reported using the wild-type of this bacterium, nevertheless acetic acid production from alginate in this bacterium has been achieved [25]. As mentioned above, the major reasons why the bacterium did not produce ethanol were due to an imbalance of reductive power in alginate metabolisms [10,12,23,26]. A sugar alcohol, mannitol, generates two molecules of NADH, but alginate generates only one molecule of NADH during metabolism [24].

The aims of this study are to create a metabolically engineered *V. halitotocoli* to compensate

105 for the reducing power and produce ethanol from alginate. To achieve this purpose, we tried to
106 introduce two genes; pyruvate decarboxylase (*pdc*) gene and alcohol dehydrogenase II (*adhII*) gene
107 both derived from *Z. mobilis* strain ZM4 [6] (Fig. S1). *Z. mobilis*, which is used in the tropics in the
108 production of pulque and alcoholic palm wines, using the Entner-Doudoroff (ED) pathway to
109 metabolize glucose, which results in only one mole of ATP being produced per one mole of glucose.
110 This bacterium was isolated from locally brewed alcohol and possesses the ability to ferment alcohol,
111 and is currently utilized commercially in the production of tequila [18]. The Pdc catalyzes the
112 irreversible conversion of pyruvate to acetaldehyde, in which NADH is not required. The Pdc has the
113 lowest *K_m* value: 4.4×10^{-3} M at pH 6.0 [27]. AdhII catalyzes the reversible reaction of acetaldehyde
114 to ethanol. The optimum pH of AdhII is pH 5.0 for acetaldehyde reduction and pH 9.5 for ethanol
115 oxidation, respectively [27]. Those genes were arranged in tandem to create a synthetic operon, which
116 is called the Pet (production of ethanol) operon, and it has already been confirmed that the expression
117 of the Pet operon enables ethanol production from glucose in *E. coli* [6]. We also expect that the
118 expression of the Pet operon saves reducing powers in the cells, and these excess reducing powers
119 might be used for ethanol production in *V. haliotocoli* [6,23-24]. Here we report the first creation of
120 new marine microbial biocatalysts based on alginolytic marine vibrios including *V. haliotocoli*
121 implemented with the Pet operon resulting in ethanol production from alginate.

122

123 MATERIALS AND METHODS

124 Bacterial Strains and the Culture

125 *E. coli* strains were grown in LB agar medium at 37°C. *V. haliotocoli* IAM 14596^T was grown
126 on ZoBell 2216E agar medium supplemented with 0.5% sodium alginate at 25°C [19]. In addition to
127 *V. haliotocoli*, seven alginolytic marine vibrios belonging to the Haliotocoli and Splendiduds clades
128 [22-23] were also used in this study. They were grown on the same medium used for the *V. haliotocoli*.
129 The *Z. mobilis* strain ZM4 possessing *pdc* and *adhII* genes was grown in YG agar medium at 30°C
130 [18,28,29].

131

132 **Construction of Recombinant *Vibrio halioticoli***

133 The *pdg* and *adhII* genes were amplified by PCR using primers 1
134 (CGCGCATGCGCAATGAGTTATACTGCT) and 2
135 (GTGTCTAGAAAAGTAGAGGAGCTTGTTAACAGGC), and primers 3
136 (ACGGCATGCGGGTGAGGTTATAGCTATG) and 4
137 (GGCTCTAGAGAAAACCGTTTTCTGTTTTG) from a chromosomal DNA of *Z. mobilis* ZM4 as
138 a template, respectively. Single PCR product was digested using *SphI* and *XbaI* and ligated into a
139 *SphI/XbaI*-treated pVSV208 [*Cm^r*, *rfp*] vector [30]. Both PCR product and vector were purified after
140 an agarose gel electrophoresis. Each gene was located under the control of *lac* promoter of pVSV208.
141 The resultant plasmid was first transformed into *E. coli* DH5 α λ -*pir* by electroporation using
142 MicroPulser according to the protocol provided by the manufacturer (BioRad, Hercules, CA), and
143 then the recombinant plasmid harboring *pdg* or *adhII* was transferred to wild-type *V. halioticoli* by a
144 triparental mating with *E. coli* strain CC118 λ -*pir* containing pEVS104 (*Km^r*, *oriT*) as a helper [31].
145 Before the triparental mating, *E. coli* colonies showing higher Pdc activity were selected based on
146 growth on LB agar plate supplemented with 2% glucose for 3 days at 37°C. Bigger colonies were
147 reported to show higher Pdc activity than normal because of increased substrate utilization rates and
148 resultant improved cell growth [6,32]. Recombinant *V. halioticoli* cells were further screened on a
149 TCBS plate at 25°C based on higher growth rates, and the selected colonies were purified on a ZoBell
150 2216E agar plate supplemented with 0.5% alginate and 5 μ g/mL chloramphenicol at 25°C. Finally,
151 positive recombinant *V. halioticoli* possessing *pdg* or *adhII* genes were confirmed using a colony PCR.
152 Enzyme activities of Pdc and AdhII were measured in the PCR positive vibrios manually (see the
153 following section Enzyme Assay for Pdc and AdhII). Pdc and AdhII expressing recombinant *V.*
154 *halioticoli* strains were named as *V. halioticoli* (pVIP208) and the *V. halioticoli* (pVIA208),
155 respectively. The recombinant plasmids were extracted and purified from those recombinant *V.*
156 *halioticoli* cells using Pure Yield kit (Promega, Madison, WI) for further steps to obtain the Pet

157 (production of ethanol) operon.

158 To obtain a plasmid containing the Pet operon containing *pdh* and *adhII* gene cassettes, an
159 overlap PCR was performed [33-34] (Fig. S2). The overlap PCR has been widely used in site-directed
160 mutagenesis or in splicing DNA fragments. The overlap PCR method consists of two rounds of PCRs.
161 In the first round, two target fragments are amplified using primers 1 and 5
162 (GTTGAAGAAGCCATAGCTATAACCTCACCCAACTAGAGGAGCTTGTTGAACAGGCTTA
163 CG) and primers 2 and 6
164 (CGTAAGCCTGTTAACAAGCTCCTCTAGTTTGGGTGAGGTTATAGCTATGGCTTCTTCAAC
165), which added overlapped sequences of about 25 bp to join two genes, and pVIA208 and pVIP208
166 as templates, respectively, and then purified. In the second-round PCR, two PCR products were
167 annealed by the overlapped sequences using a thermal cycler, and then further PCR using specific
168 primers 1 and 4 was performed to obtain the PET operon. PCR was performed using *pfu* DNA
169 polymerase (Promega) under the following conditions: 94°C for 1 min, 5 cycles (94°C for 1 min,
170 55°C for 30 s, and 72°C for 4 min). The overlap PCR product was digested with *Sph*I and *Xba*I, and
171 then ligated into *Sph*I/*Xba*I-treated pVSV208. The resultant plasmid was introduced into wild-type *V.*
172 *haliotocoli* by triparental mating in the same way as described above. The Pdc and AdhII activities of
173 the selected colonies were measured (see the section below on Enzyme Assay for Pdc and AdhII) and
174 confirmed sequences of inserted genes. Recombinant vibrios other than *V. haliotocoli* were also
175 created using the same methodology.

176

177 Enzyme Assay for Pdc and AdhII

178 *V. haliotocoli* cells were cultured in 100 mL flasks containing 50 mL of ZoBell 2216E broth
179 at 25°C with shaking (130 rpm). The cultured cells were collected by centrifugation (6,200 x g, 4°C,
180 Allegra 21R, Beckman, California), and washed once with 50 mM MES buffer (pH 7.5), suspended
181 in the same buffer, and then finally treated with the addition of a couple of drops of chloroform. After
182 centrifugation at 19,000 x g for 2 min. at 4°C, the supernatant was used as a crude enzyme fraction.

183 The Pdc assay was performed by monitoring the pyruvate-dependent reduction of NAD with alcohol
184 dehydrogenase as a coupling enzyme [28]. In the first reaction, pyruvic acid is reduced to
185 acetaldehyde, and the acetaldehyde is further reduced to ethanol accompanied by the NAD reduction
186 in the second step. These reactions were performed at room temperature at pH 6.0. AdhII assay was
187 performed by monitoring the ethanol reduction of NAD [29]. The reactions were performed at room
188 temperature at pH 10.8. Increases or decreases in absorbance at 340 nm were continuously monitored
189 using a spectrophotometer (Ultrospec2000, Pharmacia Biotech, Little Chalfont, UK). One unit of Pdc
190 or AdhII activity was defined as 1 mmol/min change of substrate. Protein concentration was measured
191 by spectrophotometer (Ultrospec2000, Pharmacia Biotech) at the absorbance of 280 nm. Specific
192 activities were measured in triplicate samples.

193

194 **Ethanol Production**

195 Recombinant strains prepared in this study were pre-cultured in a 300 mL flask containing
196 100 mL of the ZoBell 2216E broth supplemented with 5 µg/mL chloramphenicol at 25°C for 24 h.
197 A total of 1 mL pre-cultured strain was inoculated in 99 mL of ZoBell 2216E broth supplemented
198 with substrates (1.5% alginate or 1.5% mannitol), 1.5% glutathione, 0.1 M MOPS (Dojindo Lab.,
199 Kumamoto, Japan) and 5 µg/mL chloramphenicol at pH 7.5, and a batch culture for ethanol
200 production was carried out. pH controller (DT-1023P, Tokyo) was used to maintain a pH of 7.5 by
201 the addition of filter-sterilized 5 M NaOH. The batch culture was basically carried out at 20°C for
202 72 hours, and was stirred continuously using a magnetic bar and a magnetic stirrer (RO 10 power
203 IKAMAG, IKA, Staufen, Germany) at 100 rpm for 72 h. Subsamples (1 mL) were taken using a 1
204 mL syringe every 24 h under aseptic conditions.

205

206 **Analytical Methods**

207 Ethanol production was measured using gas chromatography (GC2014, Shimadzu, Kyoto,
208 Japan) with HP-B ALC column (Agilent, Santaclala, CA, USA). Organic acids were measured

209 using high-performance liquid chromatography (Shimadzu, Kyoto, Japan) with tandem Shim-pack
210 SCR-101H column system according to the manufacturer's protocol (Shimadzu). Cell growth was
211 monitored using a spectrophotometer (Ultrospec2000, Pharmacia Biotech) at 620 nm as OD₆₂₀.
212 Quantification of alginate was performed with the phenol-sulfuric acid method, using sodium
213 alginate as the standard [35].

214

215 **Sequencing of *pdc* and *adhII* genes**

216 Recombinant *V. haliotocoli* was cultured in 1,000 mL flask containing 400 mL ZoBell 2216E
217 broth containing 5 µg/mL chloramphenicol for 24h. Plasmid extraction was performed using Pure
218 Yield Plasmid Miniprep System (Promega, Madison, WI). Extracted plasmid was purified using
219 DNeasy Blood & Tissue Kit (QIAGEN, Valencia, USA) and concentrated with ethanol precipitation.
220 DNA sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit and Genetic
221 Analyzer 3130xl according to the protocol provide by the manufacturer (Applied Biosystems, Foster
222 City, CA, USA).

223

224 **Statistics**

225 Bayes statistics was used for test whether null hypothesis (H_0), which is the effect size=0,
226 was more probably occurred or not than the alternative hypothesis (H_1) based on bayes factor (BF_{10})
227 using JASP software version 0.17.1 (<https://jasp-stats.org/>).

228

229 **RESULTS and DISCUSSION**

230 Metabolic engineering has opened new doorways in material production using mainly model
231 microorganisms such as *E. coli*, *Saccharomyces* and so on [6-7,12-13,15]. In this study, we have
232 successfully engineered alginolytic marine vibrios, and have produced ethanol from alginate, which
233 is a highly oxidized polysaccharide contained in brown macroalgal biomass. To our knowledge, this
234 is the first report of successful ethanol production from alginate using recombinant marine bacteria.

235 Three types of recombinant *V. haliotocoli* strains, harboring *adhII*, *pdc*, and the Pet operon
236 (*adhII-pdc* gene cassette) were constructed. These recombinants were named as the *V. haliotocoli*
237 (pVIA208), the *V. haliotocoli* (pVIP208) and the *V. haliotocoli* (Pet), respectively. Specific activities
238 of Pdc or Adh in a wild-type *V. haliotocoli* were 0.002 U/mg protein and 0.008 U/mg protein,
239 respectively, however, the specific activities of Adh in the recombinant *V. haliotocoli* (pVIA208) and
240 Pdc in the recombinant *V. haliotocoli* (pVIP208) increased to 0.017 U/mg and 1.07 U/mg, respectively
241 (Fig. 1). The Adh activity remained at low levels in the *V. haliotocoli* (pVIA208), but the Pdc activity
242 was significantly higher than that of the wild type. Furthermore, both Pdc and Adh activities greatly
243 increased in the recombinant *V. haliotocoli* (Pet) compared to those of the recombinant *V. haliotocoli*
244 implemented by single gene, reaching 7.8 U/mg protein and 2.8 U/mg protein, respectively (Fig. 1).
245 These specific activities of Pdc and Adh in the *V. haliotocoli* (Pet) increased 3,459- and 355-folds,
246 respectively. More Pdc activity than AdhII activity was also measures in *E. coli* (Pet) [6], so similar
247 trends of the Pet operon under control of lac promoter were observed in this marine vibrio.

248 Nevertheless, no apparent ethanol peak was detected in the culture supernatant of wild-type
249 *V. haliotocoli* in a culture, apparent and reproducible ethanol production was measured in that of
250 recombinant *V. haliotocoli* (Pet) (Fig. 2AB). The ethanol production reached 880 mg/ L from 15 g/L
251 alginate as a substrate up to 72 h maximum (612±237 SD mg/L in average in triplicate samples).
252 Almost all the alginate, which corresponded to 99.999% substrates, was consumed, and only 0.2
253 mg/L alginate remained, during the culture period. The conversion efficiency of ethanol was 1%.
254 Levels of organic acid production between the engineered and wild was strains were unlikely to be
255 different, but the recombinant *V. haliotocoli* (Pet) produced slightly higher lactate (120 mg/L) than
256 that (60 mg/L) of the wild-type *V. haliotocoli* (Fig. 2C). The growth of the recombinant *V. haliotocoli*
257 was at the same level to that of wild-type, in particular, until those stationary phases (data not
258 shown). In preliminary experiments, more ethanol production by the engineered *V. haliotocoli* (Pet)
259 was observed at pH 7.5 and 20°C.

260 Seven more recombinant alginolytic vibrios (Pet) were also constructed in this study (Fig.
261 3). The recombinant *V. rarus* RW22^T (Pet) produced ethanol at 656 mg/L, followed by the
262 recombinant *V. pelagius* (Pet) (628 mg/L), *V. haliotocoli* (Pet), and the other strains (*V. comitans*, *V.*
263 *ezurae*, *V. gallicus*, *V. inusitatus*, and *V. neonatus*) (below 400 mg/L ethanol). Apparent ethanol
264 productions by recombinant vibrios from alginate rather than those of wild type strains were
265 strongly supported based on Bayes factors (BF₁₀=5483.440) (Fig. 3).

266 The Pet operon, consisting of *pdh* and *adhII* genes cassette from *Z. mobilis* ZM4,
267 developed by Ingram *et al.* (1987), is a powerful tool to create ethanologenic *E. coli* [6]. The Pet
268 operon was widely used to engineer alginolytic terrestrial microbes to be homoethanologenic
269 because of the imbalance of reducing powers between the oxidized substrate, alginate, and ethanol,
270 even under aerobic conditions in alginolytic *Sphingomonas* sp. A1, and in a dual engineered
271 alginolytic *E. coli* [12] and a yeast [13,14]. In addition, it is proved that the Pet operon also worked
272 well in not only the alginolytic *V. haliotocoli* cells but also in the other alginolytic marine vibrios
273 and was homoethanologenic. We tried to use the other *pdh* and *adhII* homologous genes from the
274 other *Z. mobilis* strain, but only genes from ZM4 show positive effects on ethanol production from
275 alginate in this marine vibrio (data not shown), so *pdh* and *adhII* genes from *Z. mobilis* ZM4 are
276 essential for creating ethanologenic vibrios. Unfortunately, engineered terrestrial microbes such as
277 *E. coli*, *Sphingomonas*, and yeast do not tolerate saline and/or marine culture conditions, so the
278 success of ethanol production using engineered marine vibrios under marine culture conditions
279 extends the uses of Pet operon in marine biofuel production. Considering the limitations of
280 freshwater use in this current global warming era, those engineered vibrios could contribute to
281 sustainable biofuel production using seawater-based media minimizing the need for freshwater in
282 achieving the future *Carbon Neutral World*.

283 In the engineered *V. haliotocoli* (Pet), more lactate was likely to be produced than in the
284 wild type (Fig. 2C), which suggests unexpectedly greater reducing powers of the engineered vibrio
285 cells leaked in pyruvate node to lactate during the alginate metabolism. Lactate leakages were also

286 observed in five more marine vibrio recombinants. Such leakage of reducing powers from a
287 pyruvate node to lactate has been frequently reported in *E. coli* [12, 36], so the construction of *ldh*
288 knockout mutants in these vibrios could be effective in bioethanol production using alginate as a
289 feedstock. In the engineered *Sphingomonas* sp. strain A1, the functioning of the Pet pathway was
290 successful under aerobic conditions with vigorous shaking [11]. These aerobic culture conditions
291 could repress the expression of *pfl* gene encoding pyruvate-formate lyase, which causes the
292 reduction of carbon flux to formate and acetate production. With the *ldh* knock out and optimum
293 expression of Pet pathway in those engineered marine vibrios, more ethanol production from
294 alginate will be examined in further studies.

295 Even in preliminary experiments, the imbalance of reducing equivalents by alginate was
296 improved to direct more ethanol productions by mannitol-supplementation in the engineered *V.*
297 *haliotocoli* (Pet), and the recombinant could produce over 3,000 mg/L ethanol from 50 g/L seaweed
298 powder. *V. haliotocoli* prefers polyG block structure in alginate substrate, so polyM and/or MG
299 random block structures are left degraded [37]. Engineered alginolytic marine vibrios belonging to
300 the Haliotocoli clade [22,23] possess similar alginolytic assimilation pathways, but only those
301 belonging to the Splendidus clade possess a polyM specific enzyme system. Combination cultures
302 using both type of alginolytic marine vibrios or in the quest for better strains showing no substrate
303 specificity in alginate metabolism could be further ways in increasing ethanol production and
304 decreasing residual substrates.

305

306 **CONCLUSION**

307 We succeeded in producing ethanol using highly oxidized alginate and seaweed as substrate and
308 feedstock, respectively, with metabolically engineered marine vibrios in a seawater-based medium.
309 This study may contribute to establishing a platform for efficient ethanol production from marine
310 biomass, and ultimately towards a more sustainable society. Using the metabolic engineering platform
311 based on marine microbial biocatalysts, new types of material productions and material recycling, e.

312 g. in the plastics industry, are also expected.

313

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323 **Author Contributions**

324 Conceptualization: TS; Research and Data analysis: YI and TS; Writing - original draft preparation:
325 YI, TS; Writing -review and editing: TS, CJ, HK, and SM; Resources: TS. All authors contributed
326 to the article and approved the submitted version.

327

328

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467

468 **Figure legends**

469 Fig. 1. Expression of AdhII (A) and Pdc (B) activities in the engineered *Vibrio halioticoli*.

470 Faint endogenous activities were measured in wild type. Bayes T-test with independent samples was
471 performed using the JASP version 0.17.1 and activities of Pet > those of *adh* or *pdc* only was set as
472 an alternative hypothesis (H_1), respectively. Alternative hypothesis was more likely to be occurred
473 by 53 and 8 folds, respectively ($N=3$). The error bar indicates standard error.

474

475 Fig. 2. Detection of ethanol in *Vibrio halioticoli* (Pet) after being cultured with alginate and the
476 fermentation profiles. A: wild type, B: *V. halioticoli* (Pet). The arrowhead indicates the peak of
477 ethanol. C: fermentation profiles.

478

479 Fig. 3. Developments of ethanol-producing marine vibrio catalysts implemented by the Pet operon.
480 X indicates mean in the box plot. Bayes T-test with independent samples was performed using the
481 JASP version 0.17.1 and ethanol production of Pet-implemented vibrios > those of wild type was
482 set as an alternative hypothesis (H_1). The alternative hypothesis was 5,486-folds more likely to be
483 occurred ($N=11$).

484

485 **Supplementary Figures**

486 Fig. S1. New pathways in the engineered *Vibrio halioticoli*.

487

488 Fig. S2. Outline of overlap PCR methodology to create Pet operon.