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Title

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

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Running title

A First Vibrio Biocatalyst to Produce Ethanol from Alginate

Abstract

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

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

INTRODUCTION

Sustainable energy production and supply are two of the critical global issues in developing a more sustainable society. To combat the negative effects on both the environment and human society due to the use of fossil fuels, the development of alternative renewable energy has been undertaken since the late 1970's after the so-called 'oil shock' era [1-3]. In particular, the last four decades could be defined as the era of biomass energy revolution; there have been a number of technical innovations in the production of ethanol, hydrogen and methane using biomass as feedstock [1-2,4-7]. Among these, bioethanol is the only fuel which is commercially available as a realistic gasoline (transportation fuel) alternative. Generally, biomass energy uses land-based biomass, such as corn, sugar cane and switch grass, as types of feedstock. This supply of biomass has caused a controversy in the food vs energy debate (FvE) [e.g. 8,9]. To overcome these limitations, there have been many attempts to use marine biomass as a feedstock for bioethanol production [8-15] because marine biomass has many advantages not only in solving the FvE issue but also in its rapid growth, lack of lignin, fewer fertilization uses, and soft texture capable of being milled and crushed by simple mechanical operations [11-12,15]. The uses of marine biomass also could contribute to balancing CO_2 in the ocean to sustain Blue Carbon [16]. Traditional aquaculture has been capable of strengthening regional seaweed production in Japan, Chile, China, Indonesia, the Philippines, South Korea, however, bioethanol production from marine biomass from alginate is extremely difficult due to the absence of traditionally matured biotechnologies in alcohol fermentation or in energy production using seaweed as feedstock [9-12,15].

Alginate is a major component of brown algae showing viscous features [10]. The structure is constructed by linear block copolymers of uronic acids; β-D-mannuronate (M) and α-L-guluronate (G). They are arranged in homopolymeric and heteropolymeric forms; poly-mannuronate (polyM) and poly-guluronate (polyG) are for homo- and MG random, respectively [15,17]. The high oxidative state of the polyuronate structure also forces the use of the Entner-Doudoroff pathway following the 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 halitoticoli 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, Halioticoli [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. halioticoli* to compensate

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

MATERIALS AND METHODS

Bacterial Strains and the Culture

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

Construction of Recombinant *Vibrio halioticoli*

The *pdc* and *adhII* genes were amplified by PCR using primers 1 (CGCGCATGCGCAATGAGTTATACTGCT) and 2 (GTGTCTAGAAAACTAGAGGAGCTTGTTAACAGGC), and primers 3 (ACGGCATGCGGGTGAGGTTATAGCTATG) and 4 (GGCTCTAGAGAAAACCGTTTTCCTGTTTTG) from a chromosomal DNA of *Z. mobilis* ZM4 as a template, respectively. Single PCR product was digested using *Sph*I and *Xba*I and ligated into a *Sph*I/*Xba*I-treated pVSV208 [Cm^r, *rfp*] vector [30]. Both PCR product and vector were purified after an agarose gel electrophoresis. Each gene was located under the control of *lac* promoter of pVSV208. The resultant plasmid was first transformed into *E. coli* DH5α *λ-pir* by electroporation using MicroPulser according to the protocol provided by the manufacturer (BioRad, Hercules, CA), and then the recombinant plasmid harboring *pdc* or *adhII* was transferred to wild-type *V. halioticoli* by a triparental mating with *E. coli* strain CC118 λ-*pir* containing pEVS104 (Km^r, *oriT*) as a helper [31]. Before the triparental mating, *E. coli* colonies showing higher Pdc activity were selected based on growth on LB agar plate supplemented with 2% glucose for 3 days at 37°C. Bigger colonies were reported to show higher Pdc activity than normal because of increased substrate utilization rates and resultant improved cell growth [6,32]. Recombinant *V. halioticoli* cells were further screened on a TCBS plate at 25°C based on higher growth rates, and the selected colonies were purified on a ZoBell 2216E agar plate supplemented with 0.5% alginate and 5 μg/mL chloramphenicol at 25°C. Finally, positive recombinant *V. halioticoli* possessing *pdc* or *adhII* genes were confirmed using a colony PCR. Enzyme activities of Pdc and AdhII were measured in the PCR positive vibrios manually (see the following section Enzyme Assay for Pdc and AdhII). Pdc and AdhII expressing recombinant *V. halioticoli* strains were named as *V. halioticoli* (pVIP208) and the *V. halioticoli* (pVIA208), respectively. The recombinant plasmids were extracted and purified from those recombinant *V. halioticoli* cells using Pure Yield kit (Promega, Madison, WI) for further steps to obtain the Pet

(production of ethanol) operon.

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

Enzyme Assay for Pdc and AdhII

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

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

Ethanol Production

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

Analytical Methods

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

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

Sequencing of *pdc* and *adhII* genes

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

Statistics

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

RESULTS and DISCUSSION

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

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

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

Seven more recombinant alginolytic vibrios (Pet) were also constructed in this study (Fig. 3). The recombinant *V. rarus* RW22^T (Pet) produced ethanol at 656 mg/L, followed by the recombinant *V. pelagius* (Pet) (628 mg/L), *V. halioticoli* (Pet), and the other strains (*V. comitans*, *V. ezurae*, *V. gallicus*, *V. inusitatus*, and *V. neonatus*) (below 400 mg/L ethanol). Apparent ethanol productions by recombinant vibrios from alginate rather than those of wild type strains were strongly supported based on Bayes factors (BF_{10}=5483.440) (Fig. 3).

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

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

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

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

CONCLUSION

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

g. in the plastics industry, are also expected.

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Author Contributions

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

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Figure legends

Fig. 1. Expression of AdhII (A) and Pdc (B) activities in the engineered *Vibrio halioticoli*. Faint endogenous activities were measured in wild type. Bayes T-test with independent samples was performed using the JASP version 0.17.1 and activities of Pet > those of *adh* or *pdc* only was set as an alternative hypothesis (H_1), respectively. Alternative hypothesis was more likely to be occurred by 53 and 8 folds, respectively (N=3). The error bar indicates standard error.

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

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

485 **Supplementary Figures**

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

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