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4 Title page

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Enhanced hydrogen production by a newly described heterotrophic marine bacterium, *Vibrio tritonius* strain AM2, using seaweed as the feedstock

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Abstract

To achieve more stable bio-hydrogen (bioH₂) production from non-food feedstocks, stable feedstock preparations of marine biomass and an efficient bioH₂ system using marine bacteria under saline conditions are two important key technologies that needed to be developed. *Vibrio tritonius* strain AM2, which was isolated from the gut of a marine invertebrate, was cultured under various conditions in marine broth (at initial 2.25% (w/v) NaCl) supplemented with mannitol, a seaweed carbohydrate, to evaluate its hydrogen production. The maximum molar yield of bioH₂ was recorded as 1.7 mol H₂/mol mannitol at pH 6 and 37°C. The mannitol-grown cells had higher yields of bioH₂ than the glucose-grown cells in the pH range 5.5-7.5. Compared to glucose, mannitol might be a better substrate for bioH₂ production using strain AM2. Fermentation product profiling revealed that strain AM2 might be utilising the formate-hydrogen pathway for bioH₂ production. Furthermore, strain AM2 was able to produce hydrogen from powdered brown macroalgae containing 31.1% dry weight of mannitol. The molar yield of hydrogen reached 1.6 mol H₂/mol mannitol contained in the seaweed feedstock. In conclusion, strain AM2 has the ability to produce hydrogen from mannitol with high yields even under saline conditions.

1. Introduction

The World Energy Outlook 2013 [1] reported that the orientation of global energy sectors is currently shifting drastically, resulting in many major importers becoming exporters and former exporter countries becoming leading centres of growth in terms of global demand. Evidently, there is an urgent need for alternative global energy sources to allow us to decrease our dependence on fossil fuels, which will inevitably be depleted and whose use is implicated in environmental pollution and climatic change. Hydrogen gas (H₂) is credited as one possible candidate with several socio-economic, technological and environmental benefits [2]. The gas offers great advantages over hydrocarbon fuels because its combustion yields 2.75 times more energy (122 kJ/g) than fossil fuels without the emission of CO₂ and also because it can be directly used to power fuel cells [3, 4]. In addition, this extremely clean and valuable energy carrier serves as an important feedstock in some industries. Finally, it is effective in detoxifying a wide range of water pollutants and is regularly used in the fertiliser and petroleum industries [3, 4, 5].

80
81 Rising energy costs in many countries [1] have accelerated research being performed locally
82 and/or internationally with the aim of finding energy sources to boost the economy. H₂, a
83 lucrative fuel alternative, is predicted to be a notable contributor to the total energy market
84 accounting for up to 10% of the market by 2025 [6]. It is also predicted to be a viable domestic
85 energy resource for use in all economic sectors and regions around the world [5]. However,
86 acquiring a sustainable alternative energy source is a daunting task. Currently, the prospect of
87 establishing a hydrogen energy economy cannot be fully realised in a sustainable way. Despite
88 their advantages, conventional technologies for H₂ production are still heavily dependent on the
89 reformation of fossil fuels, and most processes require intense energy at high temperatures
90 (>850°C) [3]. Thus, the majority of H₂ production is not conducted in a renewable and
91 environmental friendly manner, which conflicts with the initial purpose of its application. The
92 development of renewable technologies has become the primary bottleneck in many
93 conventional methods of H₂ production, including the most sustainable approach, such as
94 electrolysis [3, 6, 7]. For instance, the electrolysis approach suffers from two drawbacks: only
95 approximately 65% of the energy is efficiently captured using the latest technologies, and a high
96 electrical capacity is needed for the process to occur [8].

97
98 Currently, the best alternative seems to be biological processes, which utilise both renewable
99 feedstock and energy [9]. Biological H₂ production via fermentation is attracting a lot of interest,
100 predominantly because the H₂ production rate is significantly higher than that achieved by other
101 methods. In addition, fermentation is applicable to a wide range of complex forms of organic
102 substrates, and offers a simple system construction [4, 10]. Simultaneously, rapid advances in the
103 biofuel industry are increasing demand for an alternative feedstock that is more likely to be
104 sustainable both economically and environmentally. In 2008, 87 gigalitres of liquid biofuels were
105 produced from crops intended as food production such as corn and sugarcane [11]. The output
106 figures increased further in 2010 and have directly affected choices between various feedstocks
107 across different regions of the world [12] to meet demand and to alleviate public concerns over
108 the “food vs. fuel” issue. The choice has widened and now includes cellulosic biomass from
109 agricultural residues or the food industry, carbohydrate-rich industrial wastewater and
110 wastewater sludge [3]. With this rationale in mind, macroalgae has also recently come under
111 consideration as an ideal biomass feedstock for bioH₂ production [13, 14, 15].

112
113 Brown macroalgae, in particular, present several advantages that complement global needs for
114 both food and energy production [13, 14, 15]. Clearly, their marine-based cultivation has an edge
115 over any terrestrial biofuel crop due to the lack competition for arable land, the high rate of
116 productivity [16] and the lack of requirements for fresh water and fertiliser. Additionally, the lack
117 of lignins and low cellulose and lipid content have made sugar extraction feasible by simple
118 biorefinery processes such as milling, leaching and crushing [14, 15]. High carbohydrate and

sugar content also permit their successful conversion to various gaseous and liquid fuels. Brown macroalgae have a complex composition, and the potential breakdown of all components promises an efficient bioH₂ production. In general, alginate forms the major structural component of brown macroalgae and its utilization, which had previously been recognised as the greatest challenge to biological ethanol production, has already been achieved by the metabolically engineered *Escherichia coli* [14] and *Sphingomonas* sp. [13]. Alternately, mannitol, the most abundant storage component, can be extracted from milled macroalgae more easily than alginate [16]. This primary stored sugar can account for 20-30% of dry weight in some *Laminaria* species [17, 18, 19] and has been successfully used for bioethanol production [16, 19, 20].

The backbone of a successful fermentative process is the acquisition of a good host microorganism [2]. Seaweeds are rich in minerals and are known to contain relatively more sodium (Na⁺) than land vegetables [21]. At present, a high sodium (Na⁺) concentration has been reported to adversely affect the H₂ productivity of *E. aerogenes* and *Clostridium* species [9, 22, 23]. Oren [24] and Pierra *et al.* [25] reported that hydrogen producing microbes in saline environments were likely to be halophilic and/or halotolerant. In another words, the halophilic and/or halotolerant natures of the host microbes can provide huge advantages in bioH₂ production in solving the “food vs. fuel” and the “water vs. fuel” conflicts. Utilization of seawater, which covers more than 90% of the available water resources of the earth, is an ideal solution in the use of seaweed as feedstock for bioH₂ production if we can find suitable marine or halophilic host microbes.

In a recent screening of effective H₂ producers from seaweed carbohydrates under saline conditions, a newly-described facultative anaerobic marine bacterium, *Vibrio tritonius* strain AM2, was isolated from the gut of sea hare (*Aplysia kurodai*), which is a herbivorous marine animal [26, 27]. The vibrio showed an appropriate H₂ production in the glucose- and mannitol-supplemented batch cultures using a seawater broth without any pH controls. The bioH₂ production is an atypical characterization in the genus *Vibrio*, but *V. tritonius* is so unique to possess the bioH₂ production pathway [27]. As vibrios are phylogenetically related to *Enterobacteriaceae* [26, 28], such as *E. coli* and *E. aerogenes*, it is interesting to compare their metabolic pathways, with particular interest in the H₂-producing pathway. H₂ production by enterobacteria is commonly achieved by formate hydrogen lyase (FHL), which produces H₂ and carbon dioxide (CO₂) through formate oxidation [29, 30, 31, 32]. The majority of studies on fermentative H₂ production have investigated *Clostridium* [33, 34], as a representative of strict anaerobes and *E. coli* [35, 36] and *E. aerogenes* [23, 37], as representatives of facultative anaerobes. However, research focusing on H₂-producing marine vibrios has never been reported yet. In this study, the unexpectedly high H₂ productivity of marine vibrios cultured with glucose and mannitol under saline conditions is reported. Moreover, using the marine vibrio, 3 L scale

batch culture experiments with the aim of practical H₂ production were conducted using powdered brown macroalgae (*Saccharina sculpera*) as a feedstock under pre-determined optimum conditions. The ability of *V. tritonius* strain AM2 to utilise marine biomass in its indigenous environment to produce bioH₂ has opened up a new and promising approach to meet increasing energy needs and as a substitute for fossil fuels.

2. Material and Methods

2.1. Bacterial strain and culture conditions

V. tritonius strain AM2 used in this study was isolated from the gut of sea hare (*Aplysia kurodai*) [26, 27]. The nature of this facultative anaerobe was discussed in great detail in Sawabe *et al.* [27], but in summary the strain could utilize mannitol but could not degrade and utilise alginate. Strain AM2 was cultured in marine broth; 0.5% (w/v) polypeptone, 0.1% (w/v) yeast extract, 100 mM MOPS (Dojindo, Kumamoto, Japan), and 75% (v/v) artificial seawater composed of 30 g/L NaCl, 0.7 g/L KCl, 5.3 g/L MgSO₄ · 7H₂O, 10.8 g/L MgCl₂ · 6H₂O, and 1.3 g/L CaSO₄ · 2H₂O) and/or marine agar (marine broth containing 1.5% agar). Initially, the strain was precultured aerobically in marine broth to achieve a cell density of 1.0±0.1 OD₆₂₀ at 25°C with shaking at 130 rpm (NR-80, TAITEC, Saitama, Japan). The culture conditions remained similar throughout the study unless stated otherwise. Later, 1 mL of the pre-culture strain was added to 100 mL of marine broth containing 5% (w/v) glucose or 5% (w/v) mannitol, which was prepared in a glass bottle reactor. Batch fermentation culture was then conducted using a magnetic stirrer (RO 10 power IKAMAG, IKA, Staufen, Germany) with gentle mixing. No gases purging was conducted, so any oxygen dissolved in the medium and remaining in the headspace of the bottle was consumed during bacterial growth. The oxygen consumption in the headspace was checked by gas chromatography (see below section). The pH of the culture was maintained at pH 5.5, 6, 6.5 or 7.5 using a pH controller (DT-1023P, ABLE, Tokyo, Japan) equipped with an autoclavable electrode (FermProbe pH electrodes, Broadley-James Corp., Branford, USA) by adding 5 N NaOH or HCl. The effect of pH on the H₂ production was determined in three replicate cultures. The temperature of the culture was set to 25°C, 30°C, 37°C and 42°C to determine H₂ production speed. No added sugars were present in the marine broth used in the control culture.

2.2. Assays

The H₂ produced during fermentation was captured in an aluminium bag, and the volume was measured using water displacement and gas chromatography (GC8A or GC2014, Shimadzu, Kyoto, Japan) with a thermal conductivity detector and ShinCarbon ST column (Shinwa Chemical Industries Ltd., Kyoto, Japan). Extracellular ethanol, formate, acetate and lactate were measured by enzymatic measurement [38] using F-kit (Roche Diagnostics, Mannheim, Germany). Ethanol produced from seaweed was also measured enzymatically using an F-kit and gas chromatography with a flame ionisation detector and a HP-Blood Alcohol capillary column

(Agilent, California, USA) with He as a carrier gas. The glucose and mannitol concentrations in the medium were measured using the phenol-sulphuric acid method [39] and by HPLC with TSK gel Amide-80 (TOSOH, Tokyo, Japan), respectively. The cell density of the culture was measured as the optical density (OD) at 620 nm using a spectrophotometer (Ultrospec 2000, Pharmacia, Sweden). The molar yields of fermentative products including H₂ were analysed using a one-way analysis of variance (ANOVA) with the Tukey-Kramer test for multiple comparisons. Probabilities of less than 5% ($P < 0.05$) were considered statistically significant.

2.3. H₂ production from seaweed feedstock by *Vibrio tritonius* strain AM2

A batch culture experiment, using seaweed as a feedstock, was performed to evaluate H₂ production of strain AM2. The powdered brown macroalgae (*Saccharina sculpera*; made up of 0.5% (w/w) alginate, 31.1% (w/w) mannitol, 8.4% (w/w) protein, 1.6% (w/w) lipid, 5.0% (w/w) fibre, 18.9% (w/w) total ash and 4.5% (w/w) others) was kindly provided by Dr. Akihiko Fukushi (Hokkaido Research Organization, Fisheries Research Department, Kushiro Fisheries Research Institute, Kushiro, Japan). In this batch culture experiment, strain AM2 was cultured in 3 L of marine broth with 300 g of powdered brown macroalgae in a 5 L jar fermenter (MBE 500 ME, Eyela, Tokyo, Japan). The culture was conducted by adding 30 mL of pre-culture strain AM2, which gave an OD₆₂₀ of 1.0±0.1.

3. Results

3.1. Effects of pH on cell growth and H₂ production in *Vibrio tritonius* strain AM2

The H₂ production profiles of the newly described marine vibrio were determined using glucose and mannitol, which is a representative carbohydrate present in kelp (Fig. 1). The growth of mannitol-grown cells was consistently less than that of glucose-grown cells. The growth of strain AM2 was higher at pH 7.5 than under slightly acidic conditions (Fig. 1A). *V. tritonius* strain AM2 produced H₂ from glucose and mannitol in the pH range of 5.5-7.5 at 30°C (Fig. 1B). The volume of H₂ produced at pH 6 from both the glucose- and mannitol-grown cells peaked at 319 and 285 mmol/L reactor volume (Fig. 1B), respectively, but decreased to between 40 and 50% of the maximum values at pH 5.5. At pH 7.5, little H₂ production was observed. The volume of H₂ produced from mannitol was higher than that from glucose at pH 6.5 and pH 7.5 but was lower at pH values below 6 (Fig. 1B). No H₂ production was determined from a control culture containing no supplementation of any carbohydrates.

Calculation of the molar yields of H₂ from both substrates revealed differences when compared to the volume of H₂ produced by strain AM2 (Fig. 2). The molar yields of H₂ from mannitol were significantly higher than the yields from glucose ($P < 0.05$) in the pH range 5.5-6.5, and the cultures at pH 5.5 and 6.0 gave the maximum molar yield on both substrates: 1.2 mol H₂/mol glucose and 1.4 mol H₂/mol mannitol. In contrast, the pH dependency of the molar yields of

formate, a precursor of H₂ in the FHL pathway, revealed an opposite trend to the yields of H₂ from both glucose and mannitol (Fig. 2).

3.2. Effects of temperature on H₂ productivity of strain AM2

To determine the optimum temperature for H₂ production, H₂ production rates of strain AM2 were measured at pH 6 with a temperature range of 25°C to 42°C (Fig. 1C). The results showed that the H₂ production rates were strongly dependent on the temperature of the culture; the maximum production rate of H₂ was observed at 37°C in both substrate-grown cells, with production rates of 9.5 and 8.0 mmol/h/L from glucose and mannitol, respectively (Fig. 1C). The maximum production rates of H₂ at 37°C from glucose and mannitol were 1.5 and 1.2 times higher than those at 30°C, respectively. The H₂ production rates from glucose were higher than those from mannitol at all temperatures. At 25°C, the difference between glucose and mannitol was the largest. In the culture from glucose at 42°C, the volume of H₂ production was lower than at 25°C, and H₂ production stopped after 34.5 hours (data not shown). Finally, at pH 6 and 37°C, the molar yield of H₂ of strain AM2 reached 1.5 mol H₂/mol glucose and 1.7 mol H₂/mol mannitol in batch culture.

3.3. Effects of substrates and pH on fermentative products of strain AM2

The molar yields of fermentation products (sum of H₂ and formate, ethanol, acetate and lactate) produced by strain AM2 were compared over the pH range of 5.5-7.5 (Table 1). The total sum of molar yields of H₂ and formate, which were nearly equal to the molar yield of total formate generated by PFL, were rather stable at all pH levels, except for the glucose-grown cells at pH 5.5. The sum of the H₂ and formate yields from mannitol was higher than that from glucose ($P<0.05$) at pH 5.5, whereas these yields were not significantly different at pH 6-7.5.

Mannitol-grown cells showed significantly higher molar yields of ethanol than acetate at all pH levels examined, whereas glucose-grown cells showed approximately equal molar yields of ethanol and acetate. Low molar yields of lactate were observed in both substrates.

3.4. H₂ production from seaweed feedstock by the marine vibrio

Batch culture experiments to assess H₂ production from seaweed feedstock were carried out in a 5 L jar fermenter with a 3 L working volume (Fig. 3). The culture conditions were maintained at pH 6 and 37°C. The agitation was set at 600 rpm. Mannitol consumption started after inoculation of strain AM2 into the medium, and continued for 72 hours until the mannitol was completely consumed. The volume of H₂ produced reached 254 mmol/L reactor volume. The maximum production rate of H₂ was maintained for 24-32 hours after inoculation and reached 8.7 mmol/h/L reactor volume. The molar yield of H₂ was 1.6 mole H₂/mole mannitol, which corresponds to a 94% yield under the same conditions with the mannitol-supplemented culture with a 100 mL working volume. Ethanol production was also observed. The final ethanol

concentration reached 10.9 g/L, and the molar yield reached 1.4 mole ethanol/mole mannitol.

4. Discussion

H₂ is a potentially promising clean energy source for the future. To produce H₂ economically, we have to develop not only the infrastructure for the use of H₂-based energy, electricity, and transportation but also a stable H₂ supply system including the biocatalysts, and sustainable feedstock production. As marine biomass is one of the candidates that fulfills the criteria for creating a sustainable bioenergy production system, the authors have evaluated the potential in H₂ production of a newly described marine vibrio that is capable of fermenting seaweed carbohydrates under saline conditions.

The H₂ productivity of a newly isolated marine bacterium, *V. tritonius* strain AM2, was examined with the goal of optimization for practical large-scale production. A practical experiment using seaweed feedstock was performed in an optimized manner following a thorough assessment of a few essential parameters including the effects of substrate, pH and temperature. H₂ production and cell growth of strain AM2 were dependent on the pH of the medium. The volume of H₂ produced by strain AM2 increased at pH 6.0-7.5, whereas the cell density showed the opposite trend (Fig. 1AB). Therefore, the pH-dependent H₂ production is most likely not caused by the cell mass, but rather, the ability of strain AM2 to produce H₂ through gene expression or enzymatic activities responsible for H₂ production. Maximum molar yield and the production rate of hydrogen in *E. aerogenes* were observed at pH 6.3, which is considered to be due to the high NADH/NAD rate in the cells [37]. *V. tritonius* strain AM2 also possessed similar mechanism in pH dependent hydrogen production, but we need further evidences in redox state of the vibrio cells under the best pH for hydrogen production.

Temperature, a critical factor affecting the activities of microorganism and conversion rate of fermentation products, is significantly linked to the economic benefit of hydrogen technology. The effect of temperature on hydrogen production was investigated in the present study in the range of 25 to 42°C at pH 6.0, because *V. tritonius* strain AM2 is capable of growing at temperature between 15 to 40°C [27]. At 37°C, the bacterium showed maximum hydrogen yield. It is also confirmed that the hydrogen yields of the strain at 37°C below and above pH 6.0 were significant lowered than that at pH 6.0 (unpublished data). Growth rate of the strain AM2 at 37°C was likely to be higher than that at 30°C, this may cause the higher speed of hydrogen production.

High salt concentrations inhibit H₂ production by preventing cell activity [9, 22, 23, 24, 25]. The molar yield and production rate of H₂ by *E. aerogenes* (1.6 mol H₂/mol mannitol at maximum) markedly decreased to 1.2 and 0.8 mol H₂/mol mannitol under 2% and 3% (v/w) NaCl,

respectively [23]. There are only a few reports on H₂ production under marine conditions; one report focused on *Bacillus* sp. B2 [40]. It is widely accepted that Na⁺ concentration has a strong inhibitory effect on anaerobic processes including H₂ production [25, 41, 42]. More recently, Pierra *et al.* [25] reported the presence of a moderately halophilic biohydrogen producing microbial population in a saline sediment of a lagoon collecting salt factory wastewaters, and the majority of the population might consist of *Vibrionaceae* bacteria. The family of *Vibrionaceae* thus becomes a dominant species under moderate halophilic conditions and exhibits the highest hydrogen yields (0.9 mol H₂/mol Glc) at the highest NaCl concentration of 75 g/L [25]. As these vibrios have not been isolated yet, we were not able to compare the biohydrogen producing ability of *V. tritonius* AM2 directly to the lagoon vibrios. Vibrios are becoming important H₂ producers in halophilic conditions today. Surprisingly, the maximum molar yield of H₂ of the *V. tritonius* strain AM2 was recorded with 1.7 mol H₂/mol mannitol (initial 2.25% (w/v) NaCl) at pH 6 and 37°C. To the best of our knowledge, the maximum molar yield of H₂ produced by strain AM2 was higher than that of any other wild-type strains of facultative anaerobes under saline conditions. Unfortunately, the effects of NaCl concentration on H₂ production by strain AM2 are unlikely to be fully evaluated in this study. Under aerobic conditions, strain AM2 is capable of growth at 0.5-8% (w/v) NaCl range with the optimum growth at 3% (w/v) [27]. Thus, further studies on the effects of NaCl on H₂ production by strain AM2 are both worthwhile and necessary. Marine bacteria including vibrios possessed a variety of systems to use Na⁺ gradient to maintain stoichiometry and to power the cells on the basis of the whole genome sequencing of this strain (unpublished data). To understand how salts affect hydrogen production in the vibrios cells, global transcriptome studies may provide further insights.

Formate is a precursor of H₂ produced by FHL in most H₂-producing facultative anaerobes. Formate is either excreted by the cell through the formate channel (FocA) or metabolized to CO₂ and H₂ by FHL in enterobacteria [29, 30, 31, 32]. In strain AM2, the molar yields of H₂ and formate showed opposite trends with changes in the pH of the medium (Fig. 2), and the sum of the molar yields of H₂ and formate, which is nearly equal to that of total formate produced by PFL, was also stable at the pH range 6-7.5 (Table 1). The dependence of the production dynamics of H₂ and formate on pH indicates that strain AM2 mainly used FHL and produced H₂ by the oxidation of formate under slightly acidic conditions (pH 5.5 and 6.0).

The H₂-producing pathway using mannitol is elucidated by determining the balance of fermentative products at various levels of pH in the medium. Mannitol, which is a reduced form of hexose, can generate more molecules of NADH during glycolysis than glucose [32]. Generally, excess reducing equivalents, such as NADH, are consumed by the production of lactate, succinate, ethanol and 2,3-butanediol [32, 43]. In strain AM2, the molar yield of ethanol of mannitol-grown cells was greater than of glucose-grown cells, and the molar yield of acetate showed the opposite trend (Fig. 2 and Table 1). The shift in carbon flux from ethanol to acetate

also suggests that the mannitol-grown AM2 cells might be a physiologically reduced state. The molar yields of H₂ also showed trends similar to those of ethanol with changes in the pH of the medium (Fig. 2). In most facultative anaerobes, which use only the FHL pathway, NADH is unlikely to be used in H₂ production [30, 31]. However, the enhanced H₂ yield of the mannitol-grown cells suggests that there might be some effects on H₂ production due to changes in reducing equivalents and carbon flux when mannitol metabolism is used in the strain.

To understand carbon flux during H₂ production, the sum of the H₂ and formate yields was compared between glucose- and mannitol-grown cells at each pH of the medium. The sums of the H₂ and formate yields from glucose were significantly lower than that from mannitol ($P < 0.05$) at pH 5.5, whereas there were no significant differences between the glucose- and mannitol-grown cells at pH 6.0-7.5. Such results suggest that the carbon flux of strain AM2 during glucose metabolism might be less efficient at pH 5.5 than under the other culture conditions examined. In other words, the consistently high total sum of yields of H₂ and formate for mannitol-grown cells might indicate efficient mannitol metabolism by strain AM2. However, the amounts of mannitol consumed were lower than the amounts of glucose consumed at pH 5.5 and 6. However, both substrates (5% (w/v)) were consumed completely at pH 6.5 and 7.5, when gas production stopped (data not shown). These results indicate that there was a difference in the efficiency between H₂ production and sugar consumption of mannitol-grown cells. Therefore, more detailed analysis must focus on the metabolic and genetic control and/or feedback to understand the entire mannitol metabolism of strain AM2.

To enhance the molar yield of H₂, the carbon flux of the upstream pathway of formate and acetyl-CoA are important factors to be considered. Based on the fermentation pathway, in particular that described in *E. coli*, the disruption of lactate- and succinate-producing pathways enhances the molar yield of H₂. Inactivation of both the *ldhA* gene (lactate dehydrogenase gene) and the *frdBC* gene (fumarate reductase gene) enhanced the molar yields of H₂ from 1.08 to 1.82 mol H₂/mol glucose in *E. coli* [35, 36]. The molar yield of lactate produced from pyruvate was remarkably low at all pH levels examined for strain AM2 (Table 1). Although succinate production could not be measured here (though the production was recently determined to be tiny (below 0.1 mol/mol sugar)), strain AM2 shows good capability in not wasting excess reducing equivalents through lactate and succinate.

Finally, we succeeded in achieving practical H₂ production from powdered seaweed feedstock using a jar fermenter under pre-optimised culture conditions (Fig. 3). Our batch culture experiments demonstrate the advantages of strain AM2 as a high-yield H₂ producer from mannitol, which is the primary reserve compound of brown macroalgae under saline conditions. The batch culture experiment was carried out in a 5 L jar fermenter, with 3 L marine broth added to 300 g powdered brown macroalgae (*Saccharina sculpera*) at 31.1% (w/w). After 72 hours of

cultivation, all mannitol present in the seaweed feedstock was consumed (Fig. 3). The results show that the use of seaweed feedstock for fermentation can be realised by simple pre-treatment, such as pulverisation. When seaweed feedstock was used as a substrate, the molar yields of H₂ and ethanol could not be compared directly to the values observed in the *in vitro* culture with pure mannitol as a substrate, in which strain AM2 achieved 80% of the theoretical molar yield of H₂ by facultative anaerobes. The gap in yields might be caused by scale-up of the working volume (data not shown) or by fermentation inhibition due to various compounds contained in seaweed feedstock.

These batch culture experiments also reveal two further challenges to H₂ production from seaweed feedstock using strain AM2. One is the rather low H₂ production rate of strain AM2 compared to those of enterobacteria such as *E. coli* [32] and *E. aerogenes* [23, 37]. Improvement of the efficient culture method needs to be in order to increase the H₂ productivity. Tanisho [23] reported that the molar yield and production rate of H₂ in continuous culture are generally higher than in batch culture when the same strain is used. Designing gene-disrupted strains or the overexpression of genes responsible for H₂ production are also effective strategies [35, 36]. Therefore, the establishment of a continuous culture system is essential for technical advances. The other challenge is the further treatment of the residual organic fraction after fermentation of powdered seaweed, which contains mainly alginate, a component not consumed by strain AM2. Recently, we found that strain AM2 was capable of converting formate directly to bioH₂ (unpublished data). Therefore, we need to increase H₂ productivity in both the development of efficient utilization system of the residual fractions and the metabolic engineering of strain AM2.

5. Conclusions

There is still a lack of literature on hydrogen production from marine bacteria. In an attempt to fill the gap, hydrogen production by a marine derived bacterial strain was investigated with the concurrent objectives of optimizing culture condition which produce the most H₂. Metabolically versatile *Vibrionaceae* spp. [26] are phylogenetically related to *Enterobacteriaceae* [26, 28] which are widely known to be highly efficient H₂ producers. Similar to previous research on genera *Enterobacter*, *Clostridium* and *Escherichia*, the related *V. tritonius* strain AM2 is able to produce hydrogen in mesophilic conditions not only from mannitol, which is the major carbohydrate found in brown seaweed, but also from powdered seaweed. Interestingly, the strain AM2 has an advantage over the other genera in terms of the ability to produce significant amount of H₂ under higher saline condition (1.7 mol H₂/mol mannitol at initial 2.25% (w/v) NaCl) via the FHL pathway. In this study, we postulate that the *V. tritonius* strain AM2 carries some mechanisms of H₂ production possibly through its own genes and the gene products never been elucidated before. Overall, the current study successfully proposes the high potential of using marine bacteria for practical H₂ production from seaweed feedstock. Further studies on the

molecular mechanism of H₂ production by strain AM2 are already underway.

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Figure Legends

Fig. 1. H₂ production profiles of *Vibrio tritonius* strain AM2. Effects of pH on cell growth (A) and H₂ production (B). Effect of temperature on H₂ production of *Vibrio tritonius* strain AM2 in batch culture (C). A and B: closed bar, on glucose; open bar, on mannitol. Symbols: ●, glucose at 37°C; ○, mannitol at 37°C; ▲, glucose at 30°C; △, mannitol at 30°C; ■, glucose at 25°C; □, mannitol at 25°C. Culture conditions were set at 30°C (A and B), and pH 6 (C). n=3, error bars represent SD.

Fig. 2. Effects of pH on molar yields of H₂ and formate produced by *Vibrio tritonius* strain AM2 on glucose (A) and mannitol (B). Closed bar, molar yields of H₂; open bar: molar yields of formate. Culture conditions: temperature of the medium maintained at 30°C. n=3, error bars represent SD. Statistical significance ($P<0.05$) is indicated by letters.

Fig. 3. Practical H₂ and ethanol production from seaweed feedstock by *Vibrio tritonius* strain AM2 in 3 L batch culture. Culture conditions: pH and temperature of the medium maintained at pH 6 and 37°C, respectively. Symbols: ●, H₂ production; ○, ethanol production; ■, mannitol concentration.

Fig. 1. Matsumura et al.

Fig. 2. Matsumura et al.

Fig. 3. Matsumura et al.

Table 1. Fermentation product profiles

pH	Molar yields (mol/mol substrate)							
	Substrates							
	Glucose				Mannitol			
	H ₂ +Formate	Ethanol	Acetate	Lactate	H ₂ +Formate	Ethanol	Acetate	Lactate
5.5	1.22±0.04	0.66±0.12	0.64±0.1	0.06±0.01	1.53±0.11	0.85±0.13	0.31±0.03	0.04±0.00
6.0	1.42±0.1	0.57±0.03	0.67±0.03	0.06±0.00	1.58±0.07	0.92±0.07	0.39±0.02	0.06±0.01
6.5	1.37±0.06	0.82±0.09	0.75±0.07	0.06±0.00	1.41±0.00	1.12±0.05	0.37±0.02	0.05±0.00
7.5	1.54±0.07	0.77±0.04	0.71±0.07	0.01±0.00	1.56±0.05	1.11±0.04	0.28±0.08	0.02±0.01