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Short communication

Validation of the metabolic pathway of the alginate-derived monomer in *Saccharophagus degradans* 2-40^T by gas chromatography–mass spectrometry

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ABSTRACT

Marine macroalgae are potential resources for the sustainable production of biofuels and bio-based chemicals. Alginate, a major component of brown macroalgae, consists of two uronate monomers, which are further non-enzymatically converted to 4-deoxy-L-erythro-5-hexoseulose uronate (DEH). In several marine bacteria, DEH is known to be metabolized *via* three enzymatic steps, consisting of DEH reductase, 2-keto-3-deoxy-D-gluconate (KDG) kinase, and 2-keto-3-deoxy-phosphogluconate (KDPG) aldolase, which yields two glycolytic intermediates: D-glyceraldehyde-3-phosphate and pyruvate. However, such functions of these enzymes for the DEH pathway have rarely been experimentally validated. In the present study, the DEH metabolic pathway was investigated in *Saccharophagus degradans* 2-40^T, a marine bacterium that utilizes alginate. Through *in vitro* tests assisted by gas chromatography/mass spectrometry and gas chromatography/time-of-flight mass spectrometry, the purified enzymes were functionally confirmed and annotated as dehR, kdgK, and kdpgA, respectively. In conclusion, we report the *in vitro* validation of the metabolic pathway of DEH monomerized from alginate.

1. Introduction

Marine macroalgae have been proposed as promising alternatives to terrestrial biomass for the production of biofuels and bio-based chemicals using macroalgal carbohydrates [17,26]. The use of corn and sugar cane for biofuel production has been criticized due to competition with the food supply and changes in land use [20]. Brown macroalgae contain large amounts of alginate, laminaran, fucoidan, and cellulose [9,10,15]. Alginate is the most abundant component of brown macroalgae, comprising up to 40% of dry matter [15]. Alginate is a straight chain polysaccharide that contains α-L-guluronate (G) and β-D-mannuronate (M) as monomeric units arranged in three different configurations: poly-β-D-mannuronate (poly M), poly-α-L-guluronate (poly G), and heteropolymeric random poly M and G [3,5].

Although several marine bacteria are known to metabolize alginate [4,8,13,14,24,25], Gram-negative *Sphingomonas* sp. strain A1 is currently the only organism from which the complete alginate metabolic pathway has been detailed [20]. *Sphingomonas* sp. strain A1 takes up alginate *via* an alginate transport system and depolymerizes alginate into its unique monomers, forming 4-deoxy-L-erythro-5-hexoseulose uronate (DEH) with various endo- and exo-type alginate lyases [6]. DEH is then metabolized by three metabolic enzymes, DEH reductase, 2-keto-3-deoxy-D-gluconate (KDG) kinase, and 2-keto-3-deoxy-phosphogluconate (KDPG) aldolase, yielding D-glyceraldehyde-3-phosphate (G3P) and pyruvate [19,20].

Saccharophagus degradans 2-40^T, another marine bacterium whose genome has been fully sequenced [23], can efficiently metabolize alginate [2]. Moreover, recent studies identified the endo-type and exo-type alginate lyases of *S. degradans* 2-40^T (encoded by Alg7D and Alg17C, respectively), which are able to depolymerize alginate into DEH [11,12]. However, the potential metabolic pathway of DEH in *S. degradans* 2-40^T has not been completely explored. In order to potentially exploit the DEH metabolic pathways of *S. degradans* 2-40^T in industrial organisms through metabolic engineering, the verification and validation of putative DEH metabolic enzymes are essential.

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In the present study, we validated the functions of the *S. degradans* 2-40^T enzymes, DEH reductase, KDG kinase, and KDPG aldolase. Based on the *in vitro* activities of the enzymes, we have proposed the complete alginate metabolic pathway in *S. degradans* 2-40^T.

2. Materials and methods

2.1. Cloning

For cloning of genes encoding putative DEH reductase, KDG kinase, and KDPG aldolase enzymes, the genomic DNA of *S. degradans* 2-40^T was prepared as follows: *S. degradans* 2-40^T was cultivated for 18 h at 30 °C and 200 rpm in minimal medium consisting of 23 g/L Instant Ocean Sea Salt (Aquarium Systems, Mentor, OH, USA), 50 mM Tris–HCl (pH 7.4), 2 g/L glucose, 1 g/L yeast extract, and 0.5 g/L ammonium chloride. Genomic DNA was extracted from the culture using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol.

Genes encoding putative DEH reductase (short-chain dehydrogenase/reductase SDR, NCBI gene ID: 3965773), KDG kinase (2-keto-3-deoxygluconate kinase, NCBI gene ID: 3965772), and KDPG aldolase (2-keto-3-deoxy-phosphogluconate aldolase, NCBI gene ID: 3968651) of *S. degradans* 2-40^T were amplified by polymerase chain reaction (PCR) using primers listed in Table S1. Each PCR product was treated with *Bam*HI and *Not*I restriction enzymes and then ligated with a pET-21a plasmid treated with the same enzymes. *Escherichia coli* BL21 (DE3) was transformed with each of the resulting plasmids, and transformants were selected on Luria-Bertani agar (LBA; BD, Sparks, MD, USA) containing 50 μg/mL ampicillin.

2.2. Enzyme production and purification

For the production of target enzymes, *E. coli* transformants were cultivated in LBA at 37 °C and 180 rpm. When the absorbance of the culture at 600 nm (A_{600}) reached 0.5, 0.1 mM IPTG was added, and the culture was further incubated for 16 h at 16 °C. Cells were collected and disrupted by sonication (Branson, Danbury, CT, USA). After centrifugation of the lysate at 16,000 × *g* for 60 min, soluble supernatant was applied to a His-Trap column (GE Healthcare, Piscataway, NJ, USA), and proteins were eluted in Tris–HCl (pH 7). The enzyme eluent was concentrated using an Amicon tube (Millipore, Billerica, MA, USA). The concentrated enzyme solutions were analyzed with a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) and 12% (w/v) SDS-PAGE gel electrophoresis to determine the concentration, size, and purity of the target proteins.

2.3. Substrate preparation

As a substrate for the *in vitro* assay of DEH reductase activity, DEH was prepared as previously described [11,12]. Briefly, sodium alginate solution (2% w/v, Sigma-Aldrich, St. Louis, MO, USA) was treated sequentially with recombinant endo-type alginate lyase (Alg7D originating from *S. degradans* 2-40^T) and exo-type alginate lyase (Alg17C originating from *S. degradans* 2-40^T) to produce alginate oligosaccharides and DEH, respectively [11,12]. The reaction solution then underwent Bio-Gel P-2 size exclusion column chromatography to obtain purified DEH.

2.4. Enzyme assay of putative metabolic enzymes

For the *in vitro* analysis of DEH reductase activity, 1 mL reaction mixture was prepared with 20 mM Tris–HCl (pH 7), 800 μg/mL of purified enzyme, and either 1 mM NADH or 1 mM NADPH. Reductions in the absorbance at 340 nm was monitored for 40 min at 30 °C using a microplate spectrophotometer (Bio-Rad Laboratories, Richmond, CA, USA). The production of KDG was confirmed by thin layer chromatography (TLC) and gas chromatography/mass spectrometry (GC/MS).

To test enzyme activities of KDG kinase and KDPG aldolase *in vitro*, 800 μg/mL of each purified enzyme were sequentially added to post-reaction mixtures that contained the corresponding substrates for the target enzymes. For the reaction by KDG kinase, 2.5 mM of ATP was added in the reaction mixture as a phosphoryl donor. After incubating at 30 °C for 1 h, each enzymatic reaction mixture was quenched in boiling water for 5 min. The production of KDPG by putative KDG kinase was confirmed by TLC and GC/MS, and the production of pyruvate by putative KDPG aldolase was confirmed by gas chromatography/time-of-flight mass spectrometry (GC/TOF MS).

2.5. TLC

For quick assessment of intermediates produced in the enzymatic reaction mixtures above, TLC was performed as previously described [11]. Three microliters of each reaction product was applied to a silica gel plate 60 (Merck, Darmstadt, Germany). Metabolites were developed using an *n*-butanol-acetic acid-water (3:2:2 by volume) solution and visualized by heating the TLC plate at 130 °C for 5 min using a 10% (v/v) sulfuric acid solution in ethanol.

2.6. GC/MS and GC/TOF MS

For identification of DEH, KDG, and KDPG, each post-enzymatic reaction mixture was dried and derivatized as previously described [21]. Briefly, the aldehyde group of each reaction product was derivatized by methoxyamination using 50 μL of 20 mg/mL methoxyamine hydrochloride in pyridine (Sigma-Aldrich, St. Louis, MO, USA) at 75 °C 30 min. The volatility of the sample was increased by adding 80 μL of *N*-methyl-*N*-(trimethylsilyl)tri-fluoroacetamide (Sigma-Aldrich, St. Louis, MO, USA). The derivatized samples (1 μL) were analyzed with an Agilent 7890A GC/5975C MSD system (Agilent Technologies, Wilmington, DE, USA) equipped with a DB5-MS column (0.25 mm × 30 m, 0.25 μm film thickness; Agilent Technologies). The temperature of the GC oven was programmed as follows: 100 °C for 3.5 min, increased to 160 °C by 15 °C/min and held for 20 min, increased to 200 °C by 20 °C/min and held for 20 min, and increased to 280 °C by 20 °C/min and held for 5 min. Ionization was achieved by electron impact at 70 eV, and the temperature of the ion source was 230 °C. The mass spectra were recorded in the range of 50–500 *m*/*z* [21].

For analysis of pyruvate, an Agilent 7890A GC (Hewlett-Packard, Atlanta, GA, USA) coupled to a Pegasus HT TOF MS (Leco, St. Joseph, MI, USA) was used. This GC/TOF MS was equipped with an RTX-5Sil MS capillary column (Restek, Bellefonte, PA, USA) and an additional

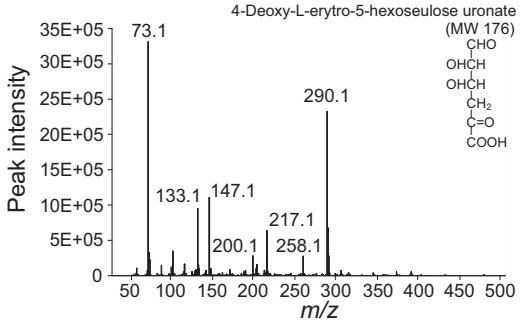

Fig. 1. Mass spectrum of the substrate, 4-deoxy-L-*erythro*-5-h*exo*seulose urinate (DEH) prepared in this study.

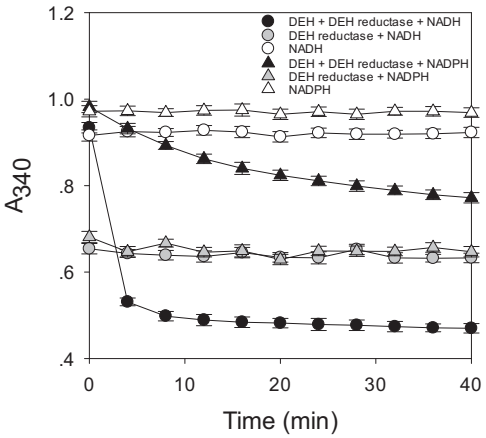

Fig. 2. Cofactor specificity of DEH reductase toward NADH and NADPH was monitored by changes in absorbance at 340 nm (A_{340}). The negative controls contained only the cofactor (NADH or NADPH) and DEH without the enzyme. All experiments were performed in triplicate.

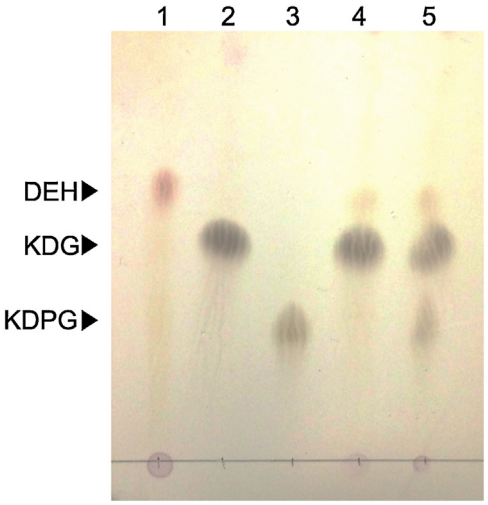

Fig. 3. TLC analysis of *in vitro* enzymatic reaction products of DEH with DEH reductase and KDG kinase. Lanes: 1, DEH standard produced from alginate by alginate lyases; 2, an authentic standard of KDG; 3, an authentic standard of KDPG; 4, the reaction product of DEH with DEH reductase; 5, the reaction product of DEH with DEH reductase and KDG kinase, introduced sequentially.

10-m integrated guard column. The temperature of the GC oven was programmed as follows: 50 °C for 1 min, increased to 330 °C by 20 °C/min and held for 5 min. The transfer line temperature was set at 280 °C. The ionization mode was subjected to an electron impact at 70 eV with an ion source temperature of 250 °C. GC/TOF MS data were preprocessed for automated peak detection by Leco ChromaTOF software (version 3.34; Leco).

3. Results and discussion

3.1. Mass fragments pattern of the substrate (DEH)

The substrate for DEH reductase activity test was in-house produced and purified following the previous report [11], in which the

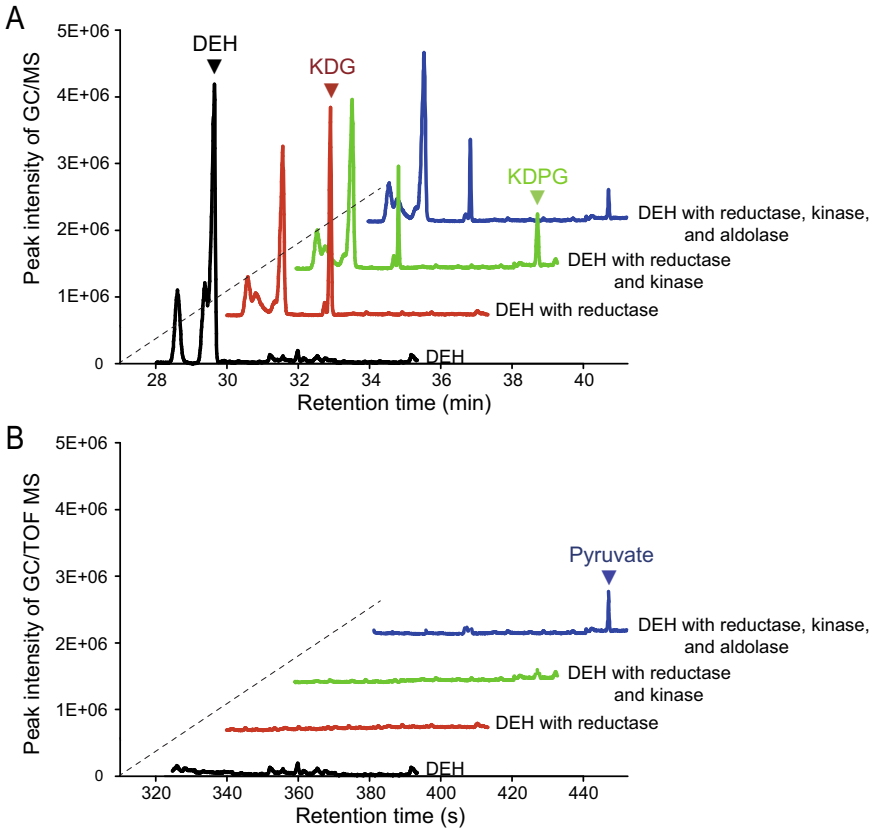

Fig. 4. GC/MS and GC/TOF MS analyses of the enzymatic reaction products. (A) Total ion chromatograms of enzymatic reaction product produced from DEH by DEH reductase, reaction product produced from DEH by DEH reductase and KDG kinase, and reaction product produced from DEH by DEH reductase, KDG kinase, and KDPG aldolase, which were analyzed by GC/MS optimized for the analysis of DEH, KDG, and KDPG. (B) Total ion chromatograms of enzymatic reaction product produced from DEH by DEH reductase, reaction product produced from DEH by DEH reductase and KDG kinase, and reaction product produced from DEH with DEH reductase, KDG kinase, and KDPG aldolase, which were analyzed by GC/TOF MS optimized for the analysis of pyruvate.

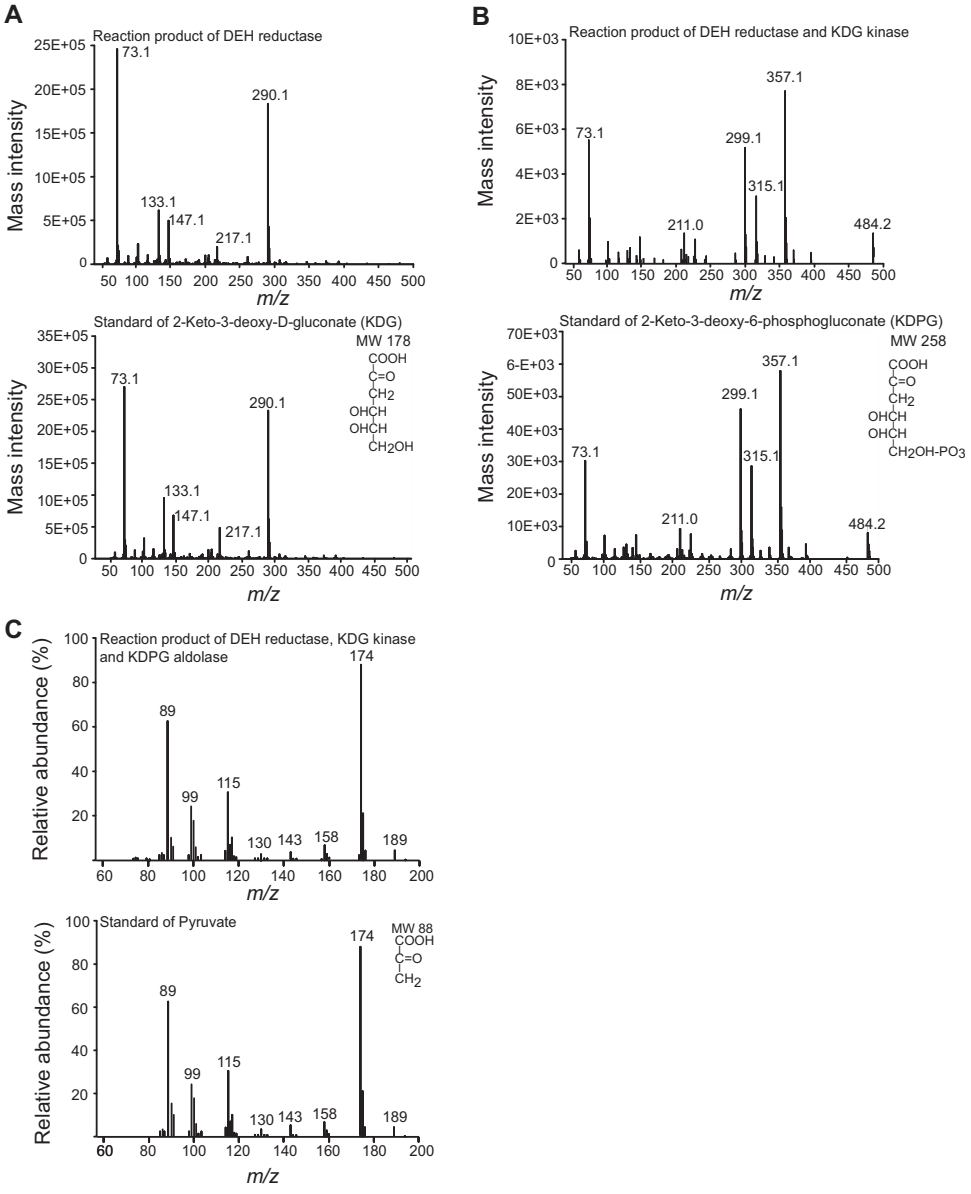

Fig. 5. Mass spectra of (A) reaction product produced from DEH by DEH reductase (upper) and an authentic standard of KDG (lower), both of which were analyzed by GC/MS; (B) reaction product produced from DEH by DEH reductase and KDG kinase (upper) and an authentic standard of KDPG (lower), both of which were analyzed by GC/MS; and (C) reaction product produced from DEH by DEH reductase, KDG kinase and KDPG aldolase (upper) and an authentic standard of pyruvate (lower), both of which were analyzed by GC/TOF MS.

same enzymes were used as in this study and the DEH was identified by liquid chromatography/mass spectrometry. In this study, the in-house prepared DEH was analyzed by GC/MS, and was found to have the same mass spectrum as that from the previous report (Fig. 1) [21]. The major fragment ions of DEH were detected at 73.1, 147. 1, and 290.1 *m*/*z*. The ions detected at 73.1 and 147.1 *m*/*z* were identified to be trimethylsilyl fragments which were derived from the derivatization process of DEH. Therefore, the ion detected at 290.1 *m*/*z* was attributed to the quasi-molecular ion of derivatized DEH.

3.2. Production of putative enzymes metabolizing DEH

In this study, we confirmed the *in vitro* enzyme activities of a putative DEH metabolic pathway of the marine bacterium *S. degradans* 2-40^T, which catabolizes alginate. Genes coding for putative DEH reductase (Sde_3281), KDG kinase (Sde_3280), and KDPG aldolase (Sde_1382) of *S. degradans* 2-40^T were individually overexpressed in *E. coli*, and the heterologous proteins were purified for *in vitro* tests. The molecular masses of the putative DEH reductase, KDG kinase, and KDPG aldolase corresponded to their theoretical mass values, 26.6 kDa, 34.8 kDa, and 21.5 kDa, respectively (Fig. S1).

3.3. Verification of DEH reductase activity and cofactor specificity

While KDG kinase and KDPG aldolase are a part of the Entner–Doudoroff (ED) pathway that many bacteria utilize [1,16,20,22], DEH reductase has been identified in only a few organisms: the *a1-R* gene of *Sphingomonas* sp. A1 [19], the *dehR* gene of *Vibrio splendidus* 12B01 [22], and the *flred* gene of *Flavobacterium* sp. UMI-01 [7]. Based on a proteomic analysis performed by a recent study, Sde_3281 of *S. degradans* 2-40^T in the alginate-specific gene cluster arose as a most promising DEH reductase [18].

To test DEH reductase activity, putative DEH reductase of *S. degradans* 2-40^T was incubated with DEH in the presence of either NADH or NADPH. During the *in vitro* enzymatic reaction, significant oxidation of NADH proceeded more rapidly than that of NADPH (Fig. 2). However, in the control experiments using reaction mixtures containing no DEH, oxidation of either NADH or NADPH was not observed. These results suggest that the putative enzyme possesses the activity of reducing DEH requiring NADH as a cofactor in preference to NADPH.

3.4. Verification of KDG kinase activity by TLC

The proposed DEH metabolic pathway of *S. degradans* 2-40^T was further verified by continuous *in vitro* enzymatic reaction of the putative DEH reductase and KDG kinase with purified DEH as a single substrate. We assumed that if the two enzymes were functional, DEH would be sequentially converted to KDG and then to KDPG. The production of KDG and KDPG were confirmed by TLC using authentic standards (Fig. 3). In lane 4 of Fig. 3, introduction of putative DEH reductase yielded a lighter band corresponding to DEH as well as a new, strong band corresponding to the KDG standard. Similarly, in lane 5 of Fig. 3, introduction of putative KDG kinase yielded a new band corresponding to the KDPG standard.

3.5. Product verification of the putative DEH metabolic pathway

To verify the *in vitro* enzymatic activities of the DEH metabolic enzymes tested above, each post-enzymatic reaction mixture was analyzed by GC/MS. Consistent with the spectrophotometric and TLC assay results described above, the first reaction mixture (putative DEH reductase) and the second post-reaction mixture (putative DEH reductase and KDG kinase) displayed new peaks that corresponded to the exact mass and mass spectra of the KDG and KDPG standards, respectively (Figs. 4a, 5a and b).

To test the third step of the DEH metabolic pathway of *S. degradans* 2-40^T, which is enabled by putative KDPG aldolase, a pre-enzymatic reaction mixture was prepared from the first and second metabolic reactions by DEH reductase and KDG kinase, respectively. Because KDPG aldolase was expected to produce pyruvate and G3P with low molecular masses, TLC would not be able to detect them. Moreover, GC/MS was not capable of identifying the product metabolites possibly due to their low amounts in the post-reaction enzymatic mixtures. To analyze pyruvate at a higher detection sensitivity, we used GC/TOF MS. As a result, pyruvate was identified from the post-reaction mixture containing DEH with putative DEH reductase, KDG kinase, and KDPG aldolase. The retention time and fragmentation pattern of the product produced by these three enzymes corresponded to those of an authentic standard of pyruvate (Figs. 4b and 5c). Taken together, our extensive analyses, including TLC, GC/MS, and GC/TOF MS analysis, verified the proposed DEH metabolic pathway of *S. degradans* 2-40^T. We also showed that the three metabolic enzymes are functional *in vitro*. All putative enzymes have now been functionally annotated: Sde_3281 for DEH reductase (DehR), Sde_3280 for KDG kinase (KdgK), and Sde_1382 for KDPG aldolase (KdpgA).

In conclusion, we report the *in vitro* validation of the metabolic pathway of DEH from alginate in a marine bacterium. Moreover, the genes encoding KDG kinase and KDGP aldolase were also verified in *S. degradans* 2-40^T, confirming the first complete DEH metabolic pathway to be revealed since that of *Sphingomonas* sp. was reported [19]. The heterologous expression of the identified DEH pathway in a robust host like *E. coli* or *Saccharomyces cerevisiae* represents the next step toward industrial algal bioprocessing. DEH metabolism in an engineered host might present several challenges, such as an inefficient expression of marine bacterial genes, cellular toxicity of DEH, and inefficient uptake of DEH. When accompanied with efficient hydrolysis of brown algae, an engineered host metabolizing DEH may be able to compete with existing crop-based bioprocesses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2016.07.020.

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