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Efficient conversion of mannitol derived from brown seaweed to fructose for fermentation with a thraustochytrid

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Macroalgae are a promising biomass feedstock for energy and valuable chemicals. Mannitol and alginate are the major carbohydrates found in the microalga *Laminaria japonica* (*Konbu*). To convert mannitol to fructose for its utilization as a carbon source in mannitol non-assimilating bacteria, a psychrophile-based simple biocatalyst (PSCat) was constructed using a psychrophile as a host by expressing mesophilic enzymes, including mannitol 2-dehydrogenase for mannitol oxidation, and NADH oxidase and alkyl hydroxyperoxide reductase for NAD^+ regeneration. PSCat was treated at 40 °C to inactivate the psychrophilic enzymes responsible for byproduct formation and to increase the membrane permeability of the substrate. PSCat efficiently converted mannitol to fructose with high conversion yield without additional input of NAD^+. *Konbu* extract containing mannitol was converted to fructose with hydroperoxide scavenging, inhibiting the mannitol dehydrogenase activity. *Auranthiochytrium* sp. could grow well in the presence of fructose converted by PSCat. Thus, PSCat is a potential carbohydrate converter for mannitol non-assimilating microorganism.

[**Key words:** Biomass; Fructose; Mannitol; Psychrophilic bacteria; NAD^+ regeneration]

Macroalgae are considered an alternative renewable feedstock for the third-generation biofuels owing to their requirement for no additional nutrients, water, and land for their cultivation, strong photosynthetic efficiency, and large carbohydrate content containing low levels of lignin (1). Macroalgae was produced 3.1 million dry metric tons of annual global production in 2006 in the world by aquaculture for algal hydrocolloids production such as alginate, agae and carrageenan (2). The macroalgae *Laminaria japonica* (*Konbu*) is the best candidate for the use as feedstock because it contains mannitol (22.5–33.2%) and alginate (14.6–29.5%) as the main carbohydrate components with mineral salts (3). We have attempted to construct a total bio-system for marine biomass utilization, including pretreatment for the decomposition of marine biomass (4,5), fermentation of methane (6–8), conversion to valuable chemicals such as unsaturated fatty acids (9), and recovery of rare metals.

Aurantiochytrium sp., a thraustochytrid marine protist, is reported to produce valuable unsaturated fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) and carotenoids such as β-carotene and xanthophylls (10,11). Unfortunately, *Aurantiochytrium* sp. cannot assimilate mannitol as the sole carbon source. Arafiles et al. (9) developed a two-stage fermentation system wherein the first stage involved the conversion of mannitol to fructose by *Gluconobacter oxydans* and the second stage involved lipid fermentation from fructose by *Aurantiochytrium* sp. Consequently, 32.1 g/L fructose was produced from 45.4 g/L mannitol in 12 h by using the *Konbu* extract, with a conversion rate of 83%, and subsequently, 18.0 mg/L of DHA was produced by *Aurantiochytrium* sp. However, as a carbon source, *G. oxydans* was also able to utilize fructose converted by mannitol dehydrogenase at the periplasm. Low yield of fructose was observed until *G. oxydans* remained in the reactor. To establish an efficient bio-conversion system, the psychrophile-based simple biocatalyst (PSCat) was constructed to supply *Aurantiochytrium* sp. with fructose. PSCat constitutes heat-treated psychrophilic cells expressing mesophilic enzymes for bio-conversion. Its use in bio-conversion has significant advantages because heat treatment at moderate temperatures inactivates psychrophilic metabolic enzymes, leading to no (or less) byproduct formation (12). Previously, we successfully applied the PSCat for the production of aspartate from fumarate without the formation of malate as a byproduct (13). The PSCat is an innovative cell catalyst for efficient bio-conversion, eliminating the complications of enzyme purification.

Mannitol 2-dehydrogenase (Mdh) is present in a wide range of microorganisms, including *Gluconobacter*, *Pseudomonas*, *Lactobacillus*, and *Leuconostoc* species. Mdh has been applied for mannitol production from fructose, since mannitol is a valuable chemical for the pharmaceutical and food industry (14–17). Mdh in a heterofermentative lactic acid bacterium *Leuconostoc pseudomesenteroides* was found to be a key enzyme for fructose reduction during

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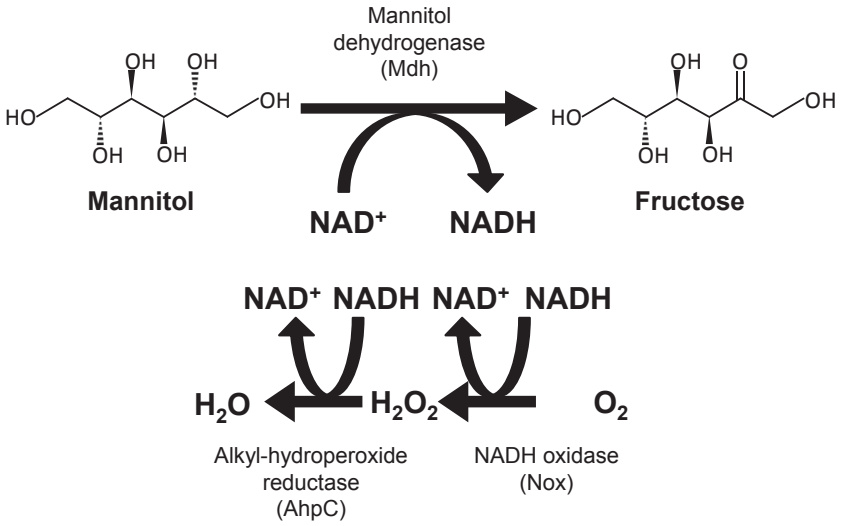

FIG. 1. Conversion of mannitol to fructose by PSCat expressing mesophilic enzymes with NAD^+ regeneration.

growth in a mixture of glucose and fructose (18). On the other hand, few studies used a reverse reaction for mannitol oxidation to form fructose. Mannitol oxidation was active at pH 8.6, although its specific activity (106 U/mg protein) was one-fourth of that in fructose reduction at pH 5.4 (400 U/mg protein) (15). The production of fructose from mannitol requires NAD^+. Hence, the regeneration of NAD^+ is required for sustainable oxidation of mannitol. NADH oxidase is a candidate for NAD^+ regeneration. Niimura et al. (19) reported an H_2O_2-forming NADH oxidase that converted NADH to NAD^+ coupled with reduction of O_2 to H_2O_2. The H_2O_2 produced was reduced to H_2O by peroxidases such as alkyl hydroperoxide reductase and catalase.

In this study, to achieve efficient fructose production from mannitol derived from the macroalga *L. japonica*, we constructed a PSCat by expressing mesophilic enzymes, including mannitol dehydrogenase of *L. pseudomesenteroides* and NADH oxidase and alkyl hydroperoxide reductase (AhpC) of *Amphibacillus xylanus* in the psychrophilic bacterium *Shewanella livingstonensis* Ac10. After the heat treatment of PSCat, fructose production from pure mannitol and *Kombu* extract was analyzed for the substrate supply to *Aurantiochytrium* sp.

MATERIALS AND METHODS

Plasmid construction The *mdh* gene was amplified by PCR from the genomic DNA of *L. pseudomesenteroides* JCM9696^T using the primers mdh_F (5′-ATGCGAATTCGGAGAGATGAACAATGGAAGCACTTGTGTTAACTGGTA-3′) and mdh_R (5′-ATGCGGTACCTTATGCCTCTTCGCCACCAACCTTA-3′) and KOD plus Neo polymerase (Toyobo, Osaka, Japan) according to the manufacturer's recommendations. PCR mixtures (50 μL) contained 10 pmoles of each primer, 200 μM dNTPs, 1.5 mM $MgSO_4$, 1 × KOD plus Neo PCR buffer, 1 U KOD plus Neo (Toyobo), and 100 ng genomic DNA. Thermocycling conditions were as follows: a denaturation step at 94 °C for 2 min, 25 cycles at 98 °C for 10 s, at 62 °C for 30 s, and at 68 °C for 40 s. The PCR fragment was digested using EcoRI and KpnI (Toyobo) and inserted into the broad-range host vector, pHA12 plasmid (20) to construct the pHA12-mdh. The cluster of *ahpC* and *nox* was amplified from 50 ng of the vector pAHNO2.5 (19) using primers ahpC_nox_F (5′-ATGCGGTACCAGGAGGAAGATGATTATGTCACTTA-3′) and ahpC_nox_R (5′-ATGCTCTAGATTAATTACGTAGTAGATAGTCAAATGCGCC-3′) and KOD plus Neo polymerase (Toyobo). Thermocycling conditions were as follows: a denaturation step at 94 °C for 2 min, 25 cycles at 98 °C for 10 s, and at 68 °C for 60 s. The PCR fragment was digested using KpnI and XbaI (Toyobo) and inserted into the pHA12-mdh plasmid to construct pHA12-mdh-ahpC-nox.

Bacterial strains and plasmids All *Escherichia coli* strains were cultured in Luria–Bertani (LB) broth or LB agar at 37 °C with 100 mg/L ampicillin as appropriate. The rifampicin-resistant mutant *S. livingstonensis* Ac10-Rif^r (21,22) was cultured in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI, USA) at 18 °C with 50 mg/L rifampicin. *E. coli* DH5α (Toyobo) was used for the construction of the recombinant plasmids. Resulting plasmids were introduced into *S. livingstonensis* by transconjugation using *E. coli* S17-1 (22). Transformed *S. livingstonensis* cells were selected using rifampicin and ampicillin at concentrations of 50 mg/L and 100 mg/L, respectively. To overexpress *mdh*, *nox*, and *ahpC*, the plasmid pHA12-mdh-ahpC-nox was introduced into *S. livingstonensis* Ac10-Rif^r, their cloned genes were introduced into the plasmid pHA12 and expressed in *S. livingstonensis* Ac10-Rif^r by induction with 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG). To prepare the overexpressing cells for the reactions, Ac10-Rif^r/mdh-ahpC-nox cells grown in 4 mL of TSB medium for two days were inoculated into 100 mL of TSB culture medium (1% inoculum). The cultures were incubated in Erlenmeyer flasks under a constant agitation of 120 rpm on a rotary shaker. After 24 h of cultivation, gene expression was induced by an addition of 100 μM IPTG. Cell growth was recorded by measuring the optical density of the culture broth at 660 nm (OD_{660}) and the dried weight of the cells.

Fructose production Cultivated cells (OD_{660} was approximately 3) were collected by centrifugation (4 °C, 5 min, 3220 ×*g*) and washed twice with 50 mM Tris–HCl buffer (pH 8.6). Before the reactions, cells were concentrated about 40 times and treated at a moderate temperature of 40 °C for 15 min. Cells (Final OD_{660} = 80) were mixed with the substrate (final mannitol concentration 50 or 75 mM). Oxygen was purged and 5 mM $ZnCl_2$ was added to the vials. The reactions were carried out at 40 °C and 120 rpm for 4 h. The supernatant was obtained by centrifugation of the reaction mixture. *Konbu* extract was prepared according to the method described in Arafiles et al. (9). Briefly, dried *Konbu* was powdered by milling machine, and the powder was diluted with milliQ water at 10% (w/v). After stirring for 20 min at room temperature and following centrifugation at 10,000 ×*g* for 20 min, *Konbu* extract was obtained as the supernatant.

Preparation of cell extract Bacteria were collected by centrifugation at 3220 ×*g* for 5 min at 4 °C and washed twice with ice-cold sonication buffer containing 100 mM Tris–HCl (pH 7.5), 20 mM KCl, 5 mM $MnSO_4$, 20 mM $MgCl_2$, 1 mM EDTA, and 2 mM dithiothreitol. The cells suspended in the same buffer were disrupted by 600 cycles of 1-s pulse/1-s intervals using an ultrasonic disintegrator (Digital Sonifier 450; Branson, Danbury, CT, USA).

Enzymatic activity Mannitol dehydrogenase activity was measured spectrophotometrically by determining the generation of NADH in the reduction reaction, as described by Kaup et al. (16). The Mdh assay mixture contained 50 mM Tris–HCl buffer (pH 8.6), 2 mM NAD^+, and 10 mM mannitol. NADH oxidase activity was measured spectrophotometrically by determining the NADH consumption. The Nox assay mixture contained 50 mM Tris–HCl buffer (pH 8.6) and 5 mM NADH. The alkyl hydroxyperoxide reductase assay was measured spectrophotometrically by determining the consumption of H_2O_2. The alkyl hydroxyperoxide reductase assay mixture contained 50 mM Tris–HCl buffer (pH 8.6) and 100 mM H_2O_2. Reaction was initiated by the addition of the crude extract, and the NADH generation or consumption of NADH, and the H_2O_2 consumption was monitored at 340 and 240 nm, respectively. The molar extinction coefficient for NADH (6300 mM^{-1} cm^{-1}) and H_2O_2 (0.0346 mM^{-1} cm^{-1}) were used to calculate the activities. One unit of Mdh, Nox and alkyl hydroxyperoxide reductase activity corresponds to the generation or consumption of 1 μmol NADH or H_2O_2 per minute.

Analysis Mannitol and fructose were quantified using a High Performance Liquid Chromatography (HPLC) equipped with an RI detector and a monosaccharide analysis column (Aminex HPX-87P, 7.8 mm ID × 300 mm L; Bio-Rad, Hercules, CA, USA) and guard column (Micro-Guard Carbo-P Refill cartridge,

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