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Bioremediation Potential of Formaldehyde by the Marine Microalga *Nannochloropsis oculata* ST-3 Strain

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Abstract The present work is intended to investigate biodegradation of formaldehyde by the marine microalga *Nannochloropsis oculata* ST-3 strain. Formaldehyde concentration in the medium decreased with the growth of the ST-3 strain. It is observed that the degradation of formaldehyde concentration depends on the increased cell number of the ST-3 strain. The ST-3 strain which was adapted to formaldehyde stepwise was able to tolerate to 19.9 ppm formaldehyde and degrade 99.3% of it in the medium for 22 days. Tolerance and degradation ability of formaldehyde by the ST-3 strain was improved by stepwise increasing of the formaldehyde concentration. Transformation of [^{13}C]formaldehyde in the medium with the passage of incubation was monitored by using a nuclear magnetic resonance (NMR) spectrometer. Formaldehyde was transformed into formate, and these two substances degraded in the medium with the passage of incubation as clearly shown by the NMR spectrum.

Keywords Formaldehyde · Marine microalga · Growth inhibition · Adaptation · Biodegradation

Introduction

Formaldehyde is widely used for the production of resins, paper, and glue in the chemical industry [1, 2]. It could be released from industrial sites during the production, use, storage, transport, or disposal of products with residual formaldehyde [1]. This wastewater eventually may be transported to the marine environment [3]. Formalin (37% formaldehyde) also is used in freshwater and marine aquaculture to control parasitic infection [4] and wash fish eggs [5]. It is also released into the marine environment from marine cultured

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finfish farms [3, 4]. Genetic damages and mutations caused by formaldehyde in microorganisms and mammals have been reported by Grafström in 1985 [6]. The long-term discharge of formaldehyde into the ocean may impact the growth and viability of a variety of marine organisms.

In order to find a viable solution to the formaldehyde problem, it is important to develop methods of removal that are both low in cost and environmental load. Recently, biodegradation was evaluated for its efficacy and applicability in the removal of formaldehyde. This technology is potentially low in cost and does not produce additional chemical hazards in the process. There are previous studies on formaldehyde biodegradation in freshwater environments. The following microorganisms have the ability to degrade formaldehyde: *Pseudomonas putida* [2, 7], *Pseudomonas cepacia* [2], *Pseudomonas alcaligenes* [8], *Pseudomonas pseudoalcaligenes* [9], *Trichosporon penicillatum* [2], *Methylobacterium extorquens* [9], and *Halomonas* sp. [10]. A few papers have been published on biodegradation of formaldehyde by bacteria which have been isolated from sea water [11, 12]. Biodegradation of formaldehyde using marine algae has not been reported.

In light of these facts, the present work is intended to investigate the degradation of formaldehyde on the marine microalga *Nannochloropsis oculata* ST-3 strain which is frequently used as a feed for rotifers [13], and metabolites of formaldehyde by the ST-3 strain.

Materials and Methods

Chemicals

[^{13}C]Formaldehyde (99 atom % ^{13}C) as a 20% solution in water and deuterium oxide (99.96 atom % D) were obtained from Isotec Inc. 2NA(EDTA·2Na) was obtained from Dojindo Laboratories. Tryptone and yeast were obtained from Becton, Dickinson and Company. All other chemicals were obtained from Wako Pure Chemical Industries, Ltd.

Algal Species and Preculture Condition of the ST-3 Strain

The test alga was marine microalga *Nannochloropsis oculata* ST-3 strain which was isolated in 2003 by Ishii et al. [14]. The ST-3 strain was inoculated in a cotton plugged 500-mL Erlenmeyer flask containing 300 mL of F/2 medium [15] without formaldehyde. F/2 medium consists of the following: 75 mg of $NaNO_3$, 6 mg of $NaH_2PO_4{\cdot}2H_2O$, 40 mg of $Na_2SiO_3{\cdot}9H_2O$, 1 mL of F/2 trace metal solution (3.16 g of $FeCl_3{\cdot}6H_2O$, 4.4 g of 2NA (EDTA·2Na), 7 mg of $CuSO_4{\cdot}5H_2O$, 7 mg of $Na_2MoO_4{\cdot}2H_2O$, 21 mg of $ZnSO_4{\cdot}7H_2O$, 12 mg of $CoSO_4{\cdot}7H_2O$, 180 mg of $MnCl_2{\cdot}4H_2O$ in 1 L of distilled water), 0.5 μg of Vitamin B_{12}, 0.5 μg of biotin, and 100 μg of thiamine HCl in 1 L of filtrated sea water. It was precultured at room temperature (24 °C) under 12:12 h light:dark cycles of 60 μE/m^2/s illumination by white fluorescent light tubes. Air permeation was sterilely conducted using a 0.22-μm sterile filter unit (SLGV 033 RS, Millipore). All the instruments and medium were sterilized using hot air sterilizer (170 °C, 150 min, WFO-600SD, EYELA) or autoclave (121 °C, 1.2 atm, 20 min, SS-235, TOMY) before experiments.

Degradation of Formaldehyde

The stock solutions were used to prepare fresh test solutions by dilution with F/2 medium. The inoculum of the ST-3 strain was taken from a preculture without formaldehyde in the

exponential growth phase to fresh test solutions. The whole volume of the medium was 500 mL in a cotton plugged 500-mL flat-bottom flask. Formaldehyde concentration in the medium was prepared at 9.9 or 19.9 ppm. The initial optical density at 720 nm (OD_{720}) of the ST-3 strain was about 0.04. The ST-3 strain was cultured in a water bath at 25 °C under 12:12 h light:dark cycles of 60 μE/m^2/s illumination by white fluorescent light tubes. Air permeation was sterilely conducted using 0.22-μm sterile filter unit. OD_{720} was measured in a spectrophotometer every 24 h for 24 days. Cell numbers and OD_{720} are highly correlated (Cell number=$(5.680\times10^7)\times OD_{720}$, $r^2=0.985$) [14]. Formaldehyde concentration in the medium was measured using an assay based on the Hantzsch reaction [16]. One milliliter of acetylacetone solution (150 g of ammonium acetate, 3 mL of acetic acid, and 2 mL of acetylacetone in 1 L of distilled water) was added to 1 mL of the sample which was filtrated by using 0.45-μm filter unit (DISMIC-25CS, ADVANTEC). After 10 min of incubation at 60 °C and cooling, the reaction product of 3,5-diacetyl-1,4-dihydrolutidine was measured by the absorbance at 412 nm. The calculations of formaldehyde concentrations were based on this absorbance at 412 nm. Bacteria-free check was conducted by using LB agar (1% (*w*/*v*)) medium containing 2.5 g of Tryptone, 1.25 g of yeast, 1.25 g of NaCl in 250 mL of distilled water throughout the experiments.

Stepwise Adaptation of Formaldehyde

Stepwise (the three steps) adaptation of formaldehyde by the ST-3 strain was investigated. The ST-3 strain was incubated at 9.9 ppm formaldehyde at the first step (Fig. 1). After 24 days of incubation, these cells were resuspended and incubated in the new F/2 medium containing 19.9 ppm formaldehyde at the second step (data not shown). After 30 days of incubation, these cells were inoculated into fresh F/2 medium of the final step adaptation test. Formaldehyde concentration in the test medium was prepared at 19.9 ppm. A test period was 22 days. Adaptation tests were performed under the same culture condition as the formaldehyde degradation test.

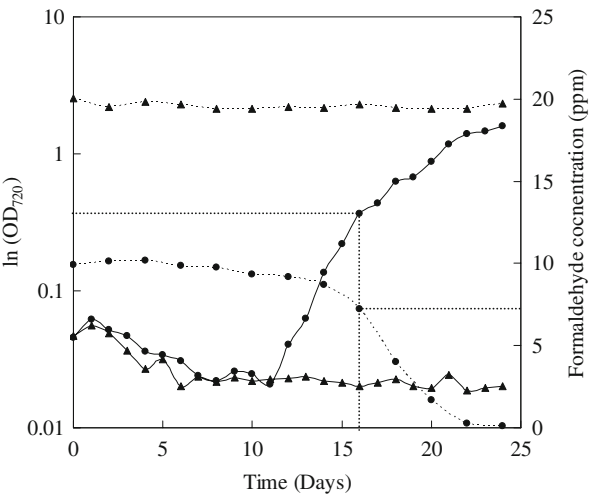

Fig. 1 Changes of cell density and formaldehyde concentration in the medium with the passage of incubation. The ST-3 strain was precultured in the absence of formaldehyde. Cell density was indicated as the optical density at 720 nm (OD_{720}). Initial formaldehyde concentration in the test medium was prepared at 9.9 (*filled circle*) and 19.9 ppm (*filled triangle*). Cell density and formaldehyde concentration are represented by *continuous* and *dashed lines*, respectively

Transformation of [^{13}C]Formaldehyde with the Passage of Incubation

Transformation of [^{13}C]formaldehyde with the passage of incubation was investigated. The ST-3 strain which was adapted up to 19.9 ppm formaldehyde was inoculated into fresh test medium containing 18.9 ppm [^{13}C]formaldehyde. Experiments were conducted under the same culture condition of formaldehyde degradation test as mentioned above. ^{13}C-nuclear magnetic resonance (NMR) spectrum of the medium filtrated by using a 0.45-μm filter unit was measured using the 500 MHz NMR spectrometer (JNM-LA500, JEOL). Data points were 32,768. The number of scans was set to 30,000 for the spectra used in the multivariate data analysis. The assays were done in the NMR tubes (φ 5 mm) with 0.6 mL of sample. A few drops of D_2O were added to provide a lock signal.

Results and Discussion

Changes of cell density and formaldehyde concentration in the medium with the passage of incubation are shown in Fig. 1. These cells were precultured in the absence of formaldehyde. The growth curve of the ST-3 strain exposed to 9.9 ppm formaldehyde showed a lag phase of 11 days. After that, ST-3 cells were able to grow at 9.9 ppm. Formaldehyde concentration in the medium began to decrease widely when OD_{720} of the ST-3 strain reached to 0.3665 (Fig. 1). Eventually, 98.7% of 9.9 ppm formaldehyde in the medium was degraded after 24 days. The ST-3 strain was able to tolerate to 9.9 ppm formaldehyde and degrade it. At 19.9 ppm formaldehyde, the growth of the ST-3 strain was not observed after 24 days. The ST-3 strain which was precultured in the absence of formaldehyde was not tolerant of the 19.9 ppm formaldehyde. Formaldehyde concentration in the medium did not decrease for 24 days. These cells were not able to degrade 19.9 ppm formaldehyde. These results indicated that the decrease of formaldehyde concentration depends on the increased cell number of the ST-3 strain.

The growth and degradation of formaldehyde by the ST-3 strain after stepwise (the three steps) adaptation up to 19.9 ppm formaldehyde are shown in Fig. 2. Formaldehyde concentration in the medium began to decrease widely when OD_{720} of the ST-3 strain reached to 0.2741 (Fig. 2) with a lag phase of 1 day. These cells were able to tolerate up to

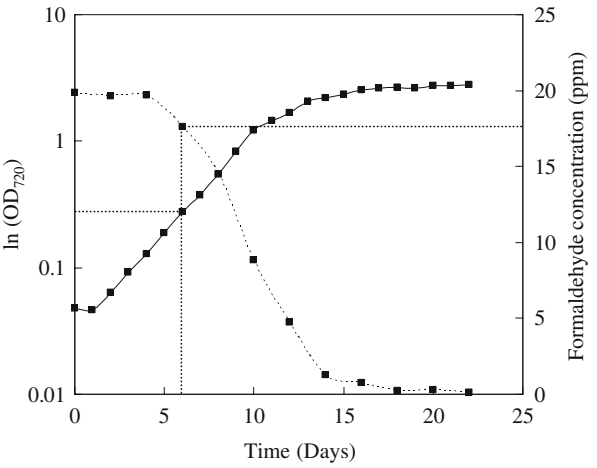

Fig. 2 Changes of cell density and formaldehyde concentration in the medium with the passage of incubation. The ST-3 strain which was adapted to formaldehyde stepwise (typically 9.9 and then 19.9 ppm) was used. Cell density was indicated as the optical density at 720 nm (OD_{720}). Initial formaldehyde concentration in the test medium was prepared at 19.9 ppm (*filled square*). Cell density and formaldehyde concentration are represented by *continuous* and *dashed lines*, respectively

19.9 ppm and degrade 99.3% of it within 22 days. Considering these results, tolerance and degradation ability of formaldehyde by the ST-3 strain was improved by stepwise increasing of the formaldehyde concentration (typically 9.9 and then 19.9 ppm). It is observed that adaptation of formaldehyde is important to degrade high levels of concentration of it. The ST-3 strain took 76 days to obtain the resistant ability of 19.9 ppm formaldehyde stepwise. We have succeeded in subculturing the ST-3 strain which was adapted to 19.9 ppm formaldehyde on agar medium. The studies on stepwise formaldehyde adaptation to microorganisms were reported previously. *Methylobacterium* strains BIP and ROS1 showed acclimatization to growth at 100 mM (≈3,000 ppm) formaldehyde [17]. *P. pseudoalcaligenes* strain OSS were able to tolerate up to 5,920 mg/L (≈5,920 ppm) formaldehyde and consumed 100% of 3,700 mg/L (≈3,700 ppm) formaldehyde after stepwise adaptation of formaldehyde [9]. Stepwise formaldehyde adaptation by marine microalgae has not been reported.

Degradation of formaldehyde by bacteria isolated from coastal sea water has been reported. The formaldehyde-resistant bacteria DM-2 strain was able to degrade 45% of 400 ppm formaldehyde within 24 h [11]. The family *Alteromonadaceae* BR-41 strain could degrade 90% of 10 ppm formaldehyde within 24 h [12]. The effectiveness of formaldehyde removal from marine environment by these bacteria was evaluated. Formaldehyde degradation ability of the ST-3 strain in this study is not so higher than these bacteria. However, the ST-3 strain can be also used as formaldehyde purification in marine environment by adaptation of higher formaldehyde concentration.

Transformation of [^{13}C]formaldehyde in the medium with the passage of incubation was investigated. The growth of the ST-3 strain and the decrease of [^{13}C]formaldehyde concentration in the medium with the passage of incubation are shown in Fig. 3. Shown in Fig. 4A,B are ^{13}C-NMR spectrum data of the medium at the first day and after 6 days. The ST-3 strain which was adapted to 19.9 ppm formaldehyde previously was used. At the first day, formaldehyde concentration was 18.9 ppm (Fig. 3), and the signal of formaldehyde hydrate $CH_2(OH)_2$ was seen at 83.0 ppm (Fig. 4B). After 6 days, formaldehyde concentration decreased to 11.8 ppm (Fig. 3), and the signal of formaldehyde at 83.0 ppm and a new signal of 172.1 ppm were seen (Fig. 4B). The ^{13}C chemical shift of 172.1 ppm represents formate HCO_2^- [18]. After 20 days, 99.6% of 18.9 ppm formaldehyde was degraded (Fig. 3), and signals of formaldehyde at 83.0 ppm and formate at 172.1 ppm disappeared (data not shown)

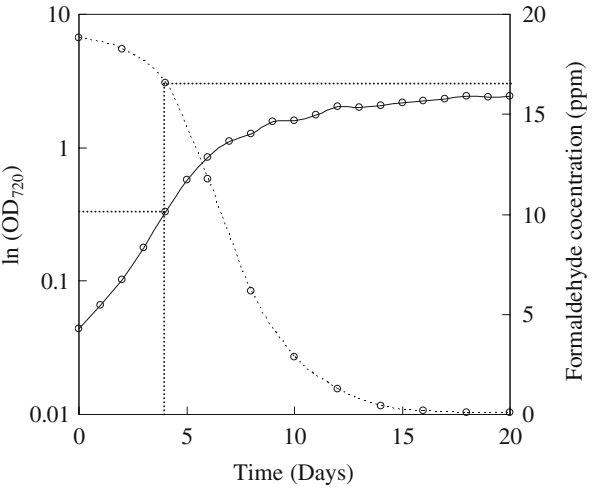

Fig. 3 Changes of cell density and [^{13}C]formaldehyde concentration in the medium with the passage of incubation. The ST-3 strain which was adapted to 19.9 ppm formaldehyde stepwise was used. The initial [^{13}C] formaldehyde concentration in the test medium was prepared at 18.9 ppm (*unfilled circle*). Cell density and formaldehyde concentration are represented by *continuous* and *dashed lines*, respectively

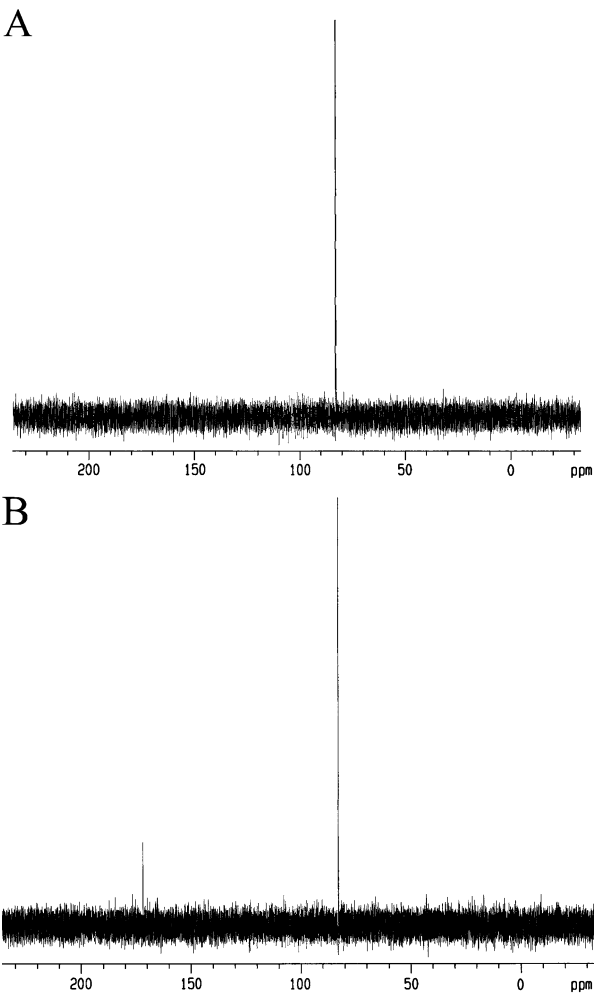

Fig. 4 The ^{13}C-NMR spectrum of the medium during incubation of the ST-3 strain for the first day (A) and after 6 days (B). Initial [^{13}C]formaldehyde concentration in the medium was prepared at 18.9 ppm. The ST-3 strain which was adapted to 19.9 ppm formaldehyde stepwise was used. The ^{13}C chemical shift of 83.0 and 172.1 ppm represents formaldehyde hydrate $CH_2(OH)_2$ and formate HCO_2^-

as well. Formaldehyde was transformed into formate, and these two substances eventually decreased in the medium with the passage of incubation. Based on these findings, the ST-3 strain has metabolized formaldehyde into formate, and has degraded formate because the signal of carbon derived from formaldehyde was not observed. These results have indicated that the ST-3 strain might have detoxicated formaldehyde in the medium.

In plants and animals, formaldehyde is metabolized and transformed to formate by glutathione-dependent formaldehyde dehydrogenase, by aldehyde dehydrogenase in mitochondria, or by catarase in peroxisome [19]. Formate is also oxidized into CO_2 by formate dehydrogenase in mitochondria and by catalase in peroxisome [20]. The ST-3 strain might have metabolized formaldehyde by these enzymes. Formaldehyde metabolism by the ST-3 strain has not been elucidated in this study. We will investigate the detailed metabolism system of formaldehyde by the ST-3 strain in the next step. This is the first study on stepwise adaptation, biodegradation, and metabolism of formaldehyde by marine microalga. Furthermore, the one of the possible applications of the ST-3 strain to remove formaldehyde in the marine environment was demonstrated in this study. Formaldehyde

could be treated by a purification biofilter of the ST-3 strain which is frequently used as a feed for rotifers in the marine aquaculture before discharge. After remediation, this alga may be used directly as the primary product in the fisheries. It is evaluated that this remediation system is both low in cost and environmental load. The ST-3 strain which has tolerance and degradation ability to high formaldehyde concentration could play an important role in the formaldehyde remediation of wastewaters from industrial sites and fisheries.

Conclusion

Biodegradation of formaldehyde by the ST-3 strain which is frequently used as a feed for rotifers in the marine aquaculture was investigated in this study. Stepwise formaldehyde adaptation was observed by the ST-3 strain. The ST-3 strain which was adapted to 19.9 ppm formaldehyde degraded 99.3% of 19.9 ppm formaldehyde in the medium. The ST-3 strain changed formaldehyde into formate. Formaldehyde and formate in the medium decreased with the passage of incubation as clearly shown by the NMR spectrum. Stepwise adaptation, biodegradation, and metabolism of formaldehyde by the marine microalga were demonstrated in this study for the first time. It is our expectation that the marine microalga will be used as bioremediation of formaldehyde in the marine environment. Assuming that the ST-3 strain can adapt to higher formaldehyde concentration, it is expected that formaldehyde used in aquaculture will be removed using the ST-3 strain.

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