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Floating Cultivation of Marine Cyanobacteria Using Coal Fly Ash

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Abstract

The aim of this study was to develop improved methodologies for bulk culturing of biotechnologically useful marine cyanobacteria in the open ocean. We have investigated the viability of using coal fly ash (CFA) blocks as the support medium in a novel floating culture system for marine microalgae. The marine cyanobacterium *Synechococcus* sp. NKBG 040607 was found to adhere to floating CFA blocks in liquid culture medium. Maximum density of attached cells of 2.0×10^8 cells/cm^2 was achieved using seawater. The marine cyanobacterium *Synechococcus* sp. NKBG 042902 weakly adhered to floating CFA blocks in BG-11 medium. Increasing the concentration of calcium ion in the culture medium enhanced adherence to CFA blocks.

Index Entries: Marine cyanobacteria; floating culture; coal fly ash; immobilization; power plant; calcium ion.

Introduction

The ability to convert CO_2 into useful materials by marine photosynthetic organisms has increased interest in their use for industrial production of chemicals *(1)* and other products *(2–7)*. Several technologies for microalgal mass cultivation in open and closed systems have been investigated *(8)*. Successful development of floating materials for immobilization of microalgal cultures can further exploit the ocean for use as a natural photobioreactor. The development of an efficient culture system is necessary for mass cultivation and industrial application of microalgae.

Some cyanobacteria and diatoms have the ability to adhere to the surfaces of materials through production of extracellular polymeric substances *(9,10)*. To develop a new method for algal cultivation, coal fly ash (CFA) blocks were manufactured to be buoyant and nutrient rich so that

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they could act as algal-supporting materials. CFA, a waste product, is collected and compressed into blocks using a resin binder. We present herein the culturing of an adhesive microalga using CFA blocks.

Materials and Methods

Strains and Culture Conditions

Marine cyanobacteria *Synechococcus* sp. NKBG 040607 and *Synechococcus* sp. NKBG 042902 were grown photoautotrophically at room temperature in either BG-11 medium *(11)* supplemented with NaCl (30 g/L), Eppley's medium *(12)*, or seawater under continuous illumination by cool white fluorescent light at an intensity of 20 μmol/($m^2 \cdot s$). BG-11 medium contained the following elements: 1.5 g of $NaNO_3$, 30 mg of $K_2HPO_4 \cdot 3H_2O$, 75 mg of $MgSO_4 \cdot 7H_2O$, 36 mg of $CaCl_2 \cdot 2H_2O$, 6 mg of citric acid, 1 mg of Na_2EDTA, 20 mg of Na_2CO_3, 6 mg of ferric ammonium citrate, 1 μg of vitamin B_{12}, and 1 mL of trace metal mix in 1 L of distilled water. Trace metal mix contained 2.86 g of H_3BO_3, 1.81 g of $MnCl_2 \cdot 4H_2O$, 222 mg of $ZnSO_4 \cdot 7H_2O$, 390 mg of $Na_2MoO_4 \cdot 2H_2O$, 79 mg of $CuSO_4 \cdot 5H_2O$, and 49.4 mg of $Co(NO_3)_2 \cdot 6H_2O$ in 1 L of distilled water. Eppley's medium contained 50.5 mg of KNO_3, 8.7 mg of K_2HPO_4, 1 mL of trace elements, and 1 mL of vitamin solution in 100 mL of distilled water and 900 mL of seawater. The trace element solution contained 19.9 mg of $CuSO_4 \cdot 5H_2O$, 44.0 mg of $ZnSO_4 \cdot 7H_2O$, 20.0 mg of $CoCl_2 \cdot 6H_2O$, 360 mg of $MnCl_2 \cdot 4H_2O$, 12.6 mg of $Na_2MoO_4 \cdot 2H_2O$, and 10 g of Fe-EDTA in 1 L of distilled water.

Determination of Microalgal Adherence to Floating CFA Blocks

CFA blocks (1 cm^3) supplemented with nitrogen (5% $NaNO_3$), phosphorus (5% $Ca_3[PO_4]_2$), and iron (1% $Fe[OH]_3$) were kindly provided by Toshiba Co. (Tokyo, Japan).

Marine cyanobacteria were cultured in 100-mL Erlenmeyer flasks containing 50 mL of BG-11 medium with one CFA block at room temperature under continuous illumination for 1 wk. Unattached cells were removed by slow rinsing using BG-11 medium. Attached cells were removed by vigorous rinsing using 5 mL of BG-11 medium. The number of cells in this latter rinse was determined by direct cell count.

Observations of Cells on CFA Block Using Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to confirm cell attachment to CFA blocks. Samples (CFA blocks with attached cells) were fixed with 5% glutaraldehyde (v/v) for 2 h at 4°C. Fixed samples were washed and incubated with 0.1 *M* cacodylate buffer (pH 7.4) for 2 h at 4°C, then soaked at 4°C with 2% osmic acid (w/v) overnight. The sample was washed several times with 0.1 *M* cacodylate buffer and then dehydrated by sequential incubation with 50, 70, and 100% (v/v) ethanol.

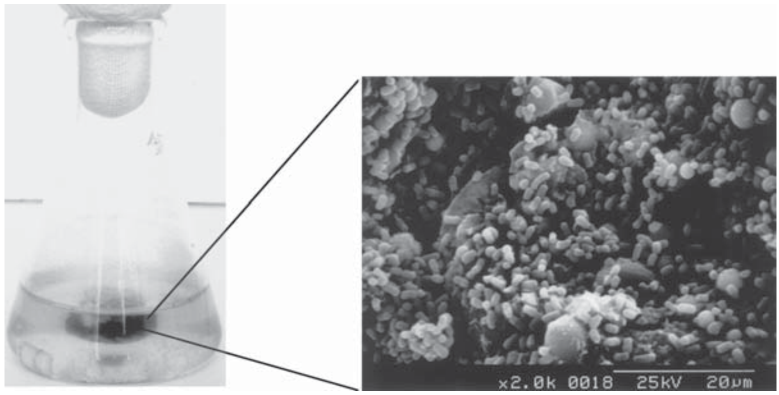

Fig. 1. Photograph of *Synechococcus* sp. NKBG 040607 grown on the CFA block.

Effect of Calcium Ion on Cell Attachment to Floating CFA Block

The effect of calcium ion on cyanobacterial adhesion to CFA blocks was investigated. BG-11 medium ordinarily contains calcium ion at a concentration of 0.25 m*M*. Modified BG-11 medium containing various concentrations of calcium at 0.25, 0.49, 0.74, 1.23, 1.72, and 2.45 m*M* was prepared. Adhesion of cyanobacterial cells to floating CFA in the modified medium was assessed as previously described.

Effect of pH on Flocculation of Cyanobacteria by Calcium Phosphate

The volume of cell suspension was adjusted to yield an effective absorbance of 8.0–9.0 at 750 nm. Adjusted 5-mL cultures were transferred to a 15-mL test tube, and 10 mg of calcium phosphate was added. The mixture was thoroughly mixed for 1 min, then left to flocculate for 5 h. Flocculation was evaluated by the flocculation rate *(13)*. The pH of the medium was adjusted to 5.0, 7.2, or 11.0 using 1 *N* NaOH or 1 *N* HCl.

Results

Adherence to a Floating CFA Block in Marine Cyanobacteria

Two marine cyanobacteria, *Synechococcus* sp. NKBG 040607 and *Synechococcus* sp. NKBG 042902, were tested for adhesion to floating CFA blocks in BG-11 medium. Maximum attached cell densities of 2.0×10^7 cells/cm^2 and 0.05×10^7 cells/cm^2 were observed after cultivation for 1 wk, respectively. Cell attachment to the surface of CFA block was confirmed using SEM (Fig. 1).

Floating Culture of Cyanobacteria with CFA Block in Various Media

Synechococcus sp. NKBG 040607 was cultured in the presence of a CFA block in seawater, BG-11, or Eppley's medium. Maximal growth was achieved using seawater (Fig. 2). The number of attached cells increased

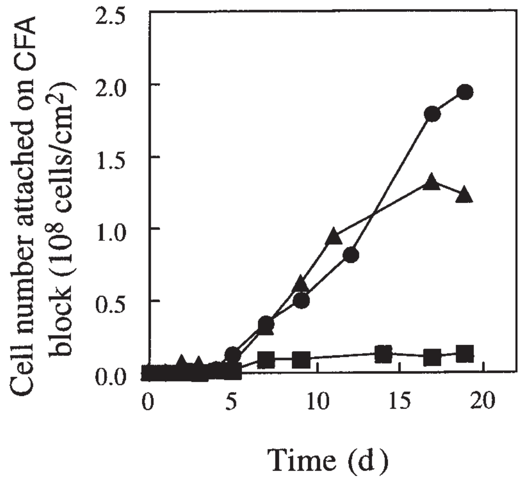

Fig. 2. Number of *Synechococcus* sp NKBG 040607 cells attached on surface of CFA block during culture. ●, Seawater; ▲, Eppley's medium; ■, BG-11 medium.

Table 1
Cell Number Attached on CFA Block
When *Synechococcus* sp. NKGB 042902 Cells Were Grown
in BG-11 Medium Containing Different Ca^{2+} Concentrations[a]

Ca^{2+} concentration (m*M*)	Cell number on surface of CFA block ($\times 10^7$ cells/cm^2)	Cell concentration in culture suspension ($\times 10^7$ cells/mL)
0.25	0.05	2.25
0.49	0.15	1.55
0.74	0.27	1.20
1.23	0.56	1.05
1.72	0.99	0.16
2.45	1.12	0.23

[a]*Synechococcus* sp. NKGB 042902 was cultured for 1 wk with CFA block.

after 4 d, reaching 2.0×10^8 cells/cm^2 after 19 d. Similar results were obtained in experiments using Eppley's medium. Cell attachment was considerably lower when using BG-11 medium.

Effect of Calcium Ion on Cyanobacterial Adherence to Floating CFA Blocks

Synechococcus sp. NKBG 042902 exhibited weak adhesion to CFA blocks floating in BG-11 medium. This strain was used to investigate the effect of calcium ion concentration on adhesion. Increasing the calcium ion concentration from 0.25 to 2.45 m*M* resulted in a 22-fold increase in attachment to CFA blocks (Table 1). This observation was accompanied by cell attachment to the bottom of the flask and a decrease in the cell concentration of the culture medium.

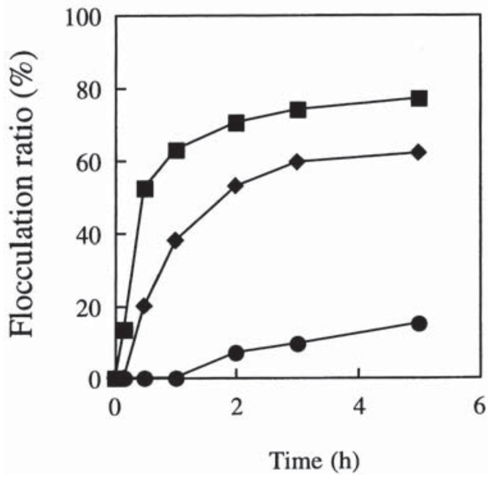

Fig. 3. Flocculation of suspension-cultured *Synechococcus* sp. NKBG 042902 by calcium phosphate ($Ca_3[PO_4]_2$). ■, pH 5.0; ◆, pH 7.2; ●, pH 11.0.

Effect of pH on Flocculation of Cyanobacteria by Calcium Ion

We hypothesize that adhesion of *Synechococcus* sp. NKBG 042902 to CFA blocks occurs through localized flocculation of cells. Calcium phosphate causes the phenomenon of flocculation or aggregation by binding with ionizable biopolymers of the cyanobacterial cell wall. Calcium phosphate is present in CFA blocks. The efficiency of flocculation using a fixed concentration of calcium phosphate can be predicted as a function of the pH. A significant correlation between pH and efficiency of flocculation using 10 mg of calcium phosphate was observed (Fig. 3). At pH 5.0, maximum flocculation (almost 80%) was observed, and at pH 7.2, intermediate flocculation efficiency was observed.

Discussion

A floating culture system using cyanobacteria was developed based on blocks manufactured from CFA. The marine *Synechococcus* sp. NKBG 040607 adhered well to CFA blocks (Fig. 1). Marine cyanobacteria can attach to surface materials through production of extracellular biopolymers *(9)*. Biosynthesis of an extracellular polymer has not yet been confirmed for this particular strain, but we hypothesize that this is the mechanism of adhesion. Seawater, a poor source of nutrients and a relatively harsh environment, yields maximum growth. Cyanobacteria can sometimes produce various substances under growth-limiting conditions. This phenomenon may have enhanced adherence.

A slightly adhesive strain, *Synechococcus* sp. NKBG 042902 in BG-11 medium, was used to investigate the effect of calcium ion on adherence. Adherence was enhanced with increasing concentrations of calcium ion when cultured in BG-11 medium. This observation suggests that calcium ion concentrations from the region 1.72 to 2.45 m*M* are sufficient to yield maximal adherence. The concentration of suspended cells was decreased with increasing calcium ion concentration. Attached cells were further observed at the bottom of the flask. *Synechococcus* sp. NKBG 042902 grown in seawater, which contains 10 m*M* calcium ion, also exhibited similar attachment tendencies (data not shown). These observations suggest that calcium ion enhances cyanobacterial adherence to CFA blocks using a slightly adhesive strain.

Calcium and magnesium ions are well-known microalgae flocculants *(14)*. They promote flocculation by attracting negatively charged algal cells in suspension. Marine photosynthetic bacteria have also demonstrated that flocculation was induced by the addition of seawater in a pH-dependent manner *(13)*. Increasing the calcium ion concentration in BG-11 medium enhanced adherence of *Synechococcus* sp. NKBG 042902. Calcium phosphate from CFA blocks, as phosphorus source, is capable of inducing flocculation in a pH-dependent manner. This observation is consistent with the hypothesis that flocculation occurs through the formation of ionic complexes between calcium phosphate and ionizable biopolymers.

CFA blocks are prepared by binding fly ash generated from the combustion of coal in power stations. The trace amount of toxic metals such as Hg, Pb, Cd, and Cr, is known to be contained in coal fly. In the case of CFA blocks proposed for use in this study, the leakage of heavy metals is significantly lower than that from untreated CFA, and complies with Japanese Industrial Standard (JIS) standards on environmental pollution. However, the impact of any such toxicity must be determined by separate studies for the bulk introduction into the open oceans.

Environmental damage, as a result of metabolites produced by cultured foreign organisms, is yet another problem that must be evaluated. Many species of cyanobacteria can produce toxins *(15–17)*. Strains must therefore be carefully selected for usefulness and nontoxicity. *Synechococcus* sp. NKBG 040607 and *Synechococcus* sp. NKBG 042902 may be used for industrial production of glutamate *(18,19)* and a plant growth regulator *(7)*, respectively. The latter strain shows calcium-dependent adherence to CFA blocks. Both strains are good candidates for immediate cultivation in the open ocean on floating CFA blocks.

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