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A fullerene colloidal suspension stimulates the growth and denitrification ability of wastewater treatment sludge-derived bacteria

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HIGHLIGHTS

- Fullerene, routinely considered as bacteriostat, was found having stimulatory effect.
- Fullerene enriched several bacteria in a denitrifying microbial community.
- Growth and metabolic activities of a *Bacillus* isolate were promoted by fullerene.

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ABSTRACT

Fullerene (C_{60}) is a nanoparticle that has been widely studied and applied in numerous commodities. However, there are concerns regarding its potential negative impact on the environment. A fullerene colloidal suspension (nC_{60}) is known for its property of selectively inhibiting the growth of microorganisms. In this study, using denaturing gradient gel electrophoresis fingerprinting technology, we found that fullerene altered the structure of a sludge-derived microbial community. Specifically, the bacteria from *Bacillus*, *Acidovorax* and *Cloacibacterium* genera were enriched in abundance when supplemented with nC_{60} at pH 6.5 under aerobic conditions. The effects of the fullerene colloidal suspension on a strain of *Bacillus* isolated from the same microbial community were evaluated to further characterize the growth-stimulating effect of nC_{60}. The biomass of cultures of this strain incubated with nC_{60} concentrations ranging from 3 mg L^{-1} to 7 mg L^{-1} was approximately twice that of the control during the stationary phase. The fullerene also induced higher superoxide dismutase activity in *Bacillus cereus*. Furthermore, the nitrate removal rate of *B. cereus* increased to nearly 55% in the presence of 5 mg L^{-1} nC_{60}, compared to 35% for the control. Meanwhile, the cumulative loading amount of nitrite was reduced from 33 μg mL^{-1} to 25 μg mL^{-1} by the addition of 5 mg L^{-1} nC_{60}. Our results demonstrate that the fullerene colloidal suspension is conditionally capable of promoting the growth and denitrification metabolism of certain bacteria, such as *B. cereus*. Fullerene might have both inhibitory and stimulatory effects on microorganisms in various environments.

1. Introduction

Fullerene (C_{60}) has been studied comprehensively and in considerable depth since its discovery by Kroto et al. (1985). Because of its signature buckyball structure and unique physicochemical properties, fullerene has been applied in the production of promising commodities, including semiconductors (Voz et al., 2007), therapeutics (Bakry et al., 2007), skin care products (Lens, 2009) and sensors (Sherigara et al., 2003). The increasing utilization of these materials and the processes related to the manufacture, usage and disposal of fullerene have resulted in their accumulation in the environment, especially in aqueous environments (Dhawan et al., 2006). Thus, evaluating fullerene's potential toxicity and characterizing its interactions with living organisms have become areas of interest in recent years. Concerns regarding its potentially detrimental effects on the health of the environment and biosphere have been raised because the implications of interactions between C_{60} and organisms remain unclear. A fullerene water suspension (nC_{60}) derived from the inerratic arrangement of single molecules is the most environmentally relevant form of fullerene in aqueous circumstances (Brant et al., 2005; Fortner et al., 2005).

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Previous studies concluded that pristine C_{60} causes no acute or sub-acute damage to assorted living organisms (Andrievsky et al., 2005; Gharbi et al., 2005; Kolosnjaj et al., 2007). Nevertheless, the potential toxicity of nC_{60} remains debatable. With respect to large eukaryotes exposed to nC_{60}, lipid peroxidation in the brain of largemouth bass (Oberdörster, 2004) and genotoxicity in human lymphocytes (Dhawan et al., 2006) have been observed. Studies on certain microorganisms showed that C_{60} could inhibit the growth of *Pseudomonas putida* (Fang et al., 2007) and could alter global gene expression of *Salmonella typhimurium* (Hancock et al., 2012) and abate ethanol production of *Zymomonas mobilis* (An and Jin, 2012). However, growth yield of *Saccharomyces cerevisiae* was not affected by nC_{60} (Hadduck et al., 2010). Some studies evaluating the toxicity of nC_{60} against *Escherichia coli* showed that nC_{60} impeded the growth of *E. coli* (Fortner et al., 2005; Chae et al., 2009), while others found that nC_{60} did not affect the growth of *E. coli* under certain conditions (Hadduck et al., 2010; Dai et al., 2012). The bacteria present in the mucus of common carp can be classified as reactive species and nonreactive species according to the amount of intracellular ROS altered by nC_{60} (Letts et al., 2011). One predictable explanation for the inconformity of biological effect of nC_{60} is that the strains used in the tests were different and thus yielded contradictory results. Therefore, further examination of the biological activity of nC_{60} should use a greater range of microorganism species to obtain comprehensive information regarding its toxicity. The objective of the present study was to evaluate the biological effects of nC_{60} on bacteria by assessing their growth and metabolism rates after incubation with nC_{60}. Previous studies on primitive form of fullerene found that pristine C_{60} could increase the life expectancy of rats (Baati et al., 2012) and promote the growth of bone cells (Zanello et al., 2006; Vandrovcova et al., 2008). However, no similar impact of nC_{60} on microorganisms has been reported. In our study, structural changes in a microbial community showed that nC_{60} had a positive effect on growth, and this effect was validated by demonstrating an increase in the biomass of *Bacillus cereus* incubated with nC_{60}.

2. Materials and methods

2.1. Preparation of the water-soluble nC_{60} suspension

Forty milligrams of pristine C_{60} powder (MER, Arizona, US) was dissolved in 100 mL toluene (Merck KGaA, Germany) by continuous shaking for 2 h at 150 rpm. Then, the toluene-soluble C_{60} solution was added to 400 mL deionized water (Milli-Q water filtration system, Millipore, Germany). The mixture was ultrasonically treated with intermittent oscillation until the majority of the toluene had been volatilized, and the solution was further treated with rotary evaporation (Buchi Rotavapor, Buchi Labortechnik AG, Flawil, Switzerland) to remove residuary toluene, followed by filtration through a 0.22 μm disposable sterile filter (Millipore, Germany). The concentration of nC_{60} was measured based on the method of Fortner et al. (2005). Briefly, 1/2 volume (1 mL) of 0.1 M $Mg(ClO_4)_2$ as destabilizer and one volume (2 mL) of toluene as a separate phase were added to one volume (2 mL) of nC_{60}. These components were mixed and frozen for 30 min. The unfrozen organic phase (toluene) was used to measure the absorption spectrum at 336 nm, the length at which C_{60} has its maximum specific absorption peak.

2.2. Sampling from the bioreactor

The microorganisms used in this present study were obtained from a laboratory scale bioreactor, which was originally built using active sludge from a wastewater treatment plant as seed sludge and contained biofilm that floated in the fluid or attached to hollow plastic carriers. The bioreactor is a continuous system, filled with plastic carries as support for bacterial biofilm development. The bioreactor is used for degrading quinolone (average 70% removal rate) and removing nitrate (average 90% removal rate) (Zhang et al., 2011). The system was predominated by facultative anaerobic bacteria. The microbial suspension sample was collected by drawing approximately 40 mL liquid (including floating biofilm and free-living microorganisms) from different parts of the bioreactor. This liquid was mixed with approximately 2 g of biofilm sample which was scratched off from the surfaces of plastic carriers with a sterile tweezer. The mixed sample was placed into a sterile centrifuge tube with glass beads. The suspension was sufficiently homogenized by using alternating steps of vortex mixing for 5 min and cooling in an ice bath for 5 min for a total of three cycles. The suspension was then centrifuged at 2000*g* for 5 min to precipitate the unhomogenized biofilm fragments. The resulting homogenized supernatant was well distributed and used as the inoculum for subsequent experiments.

2.3. Batch cultivation of the microbial community

Two hundred microliters of microbial supernatant was inoculated into 50 mL serum bottles containing 20 mL minimal Davis (MD) medium (Fortner et al., 2005) supplemented with 5 mg L^{-1} nC_{60}. Four groups of experiments were set up under different culture conditions, including aerobic groups (pH = 6.5 or 7.5) and anaerobic groups (pH = 6.5 or 7.5). For anaerobic experiments, the serum bottles were sealed with rubber stoppers and aluminum caps and were filled with N_2 (0.3 MPa, 20 s). All of the serum bottles of the four groups were incubated at 37 °C with shaking at 180 rpm for up to 7 d. Small aliquots (1 mL) of the microbial cultures from each group were collected at 1, 2, 3 and 7 d after the start of incubation. All samples were stored at −20 °C for subsequent analysis.

2.4. DNA extraction and PCR amplification

Pretreatment of the microbial culture samples and extraction of DNA were based on the method described by Zhang et al. (2010). The extracted DNA was used as template to amplify the V3 region of the 16S rRNA genes (Muyzer et al., 1993). All of the amplicons were quantified and adjusted to 30 ng μL^{-1} for use in denaturing gradient gel electrophoresis (DGGE).

2.5. DGGE analysis of the bacterial community

The 16S rDNA V3 region amplicons were analyzed on an 8% (wt/vol) polyacrylamide gel with a denaturing gradient from 24% to 54%. The electrophoresis was performed at 200 V and 60 °C for 4 h. The gels were then stained with SYBR Green I (Amresco, Solon, Ohio) and photographed with a UVI gel documentation system (UVItec, Cambridge, UK).

2.6. Identification of sequences corresponding to the DGGE band

Seven predominant DGGE bands were excised from the gels and incubated in 100 μL of sterilized distilled water overnight at 4 °C. Using 5 μL of the liquid as template, the bands were re-amplified with the aforementioned primers and program (Muyzer et al., 1993). The PCR products were purified with a DNA Purification Kit (Geneaid Biotech, Taipei) and ligated into the pGEM-T easy vector (Promega, Madison, WI). The ligations were then transformed into competent *E. coli* DH5α cells. Positive clones were identified by screening for inserts of the expected size and correct DGGE migration position. Three clones were randomly picked from the

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