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Abstract

In this study, biomineralization of covellite (CuS) and biodegradation were found at oily hot springs, in Japan. Filamentous bacteria as oil degrading bacteria and coccus typed bacteria as sulfate reducing bacteria were observed on the crude oil droplets. Bacterial cell wall had a double membrane and was surrounded by Extracellular Polymeric Substance (EPS) which served as the nucleation sites for CuS. The microbial mats contained phospholipids (lecithin and phosphoric ester), suggesting on the interaction between cell wall and oil droplets under extreme anaerobic conditions of the hot spring water. The increasing relative quantities of C_{30} hopane and the absence of pristine, phytane and trepan indicate the evaporation, leading to biodegradation. Moreover, diasteran and regular sterane ratio, Ts/Tm and oleanane/C_{30} hopane ratio also indicate that heavily biodegradation of crude oil, collected from oily hot springs, has taken place. In contrast, oil accumulation of diatom cell has been found in the reddish brown microbial mats. Two types of oil accumulation were found; encrusted with oil on the surface of cells, and accumulated oil in internal cells, associated with coccus typed bacteria, iron bacteria and green algae.

The biomineralization of covellite and the biodegradation of crude oil as described in this study could have profound implication for bioremediation of not only oil-contaminated site but also heavy metal-polluted area.

Introduction

Technological development and increasing industrial activities in the world have necessitated a vast increase in the use of fuel oil. Over the entire lifecycle including extraction, transportation, refining, storage, usage and ultimate disposal, there is a considerable risk of environmental contamination by these non-aqueous phase liquids composed of a large number of hazardous and toxic constituents. Whereas, Biomineralization has been reported in 56 phyla

of living organisms including bacteria, protozoans, fungi and plants and has, in a large number of cases, an important role in the remediation of heavy metal.

Hence, The focus of this study is to observe the effect of the green microbial mats on the removal of crude oil. Biomineralization of covellite (CuS) on the surface of crude oil droplets was observed in this study by microscopic techniques, phospholipid analysis, bacterial cultivation and GC-MS/GS techniques. In this integrated program, studies on microbial diversity and activities in response to heavy metals and oil pollution are combined with studies on behavior of micro-organic mineralization attendant on the degradation of hydrocarbon molecules in microbial systems. Knowledge of the biodiversity of microorganisms and the identification of mineral compositions, oil sensitivity, oil tolerance and oil loving species and communities may help to select effective strategies for the bioremediation of heavy metals and oil-polluted areas.

Results and discussion

Biomineralization of covellite in Tsukioka hot springs

The biomineralization of covellite in oily hot springs was found at the Tsukioka Hot Springs, Niigata, which formed in green microbial mats. Water quality of hot springs was nearly neutral pH (7.3) and anaerobic condition (Eh -184 mV). The microbial mats contained a large amount of Cu to form Cu-minerals, such as covellite (CuS). The crude oil contained high S with traces of Si and Cu, and emitted the dark orange colored fluorescence light under ultraviolet ray.

Optical and epifluorescence microscopic observations showed that filamentous bacteria and coccus typed bacteria which resided around the oil droplets and formed colonies with the yellow colored mineral particles may be covellite. Oil droplets were covered with filamentous bacteria in the biofilms formed as a result of bacterial cell lysis (Fig. 1). TEM observation revealed that filamentous bacteria had double membrane

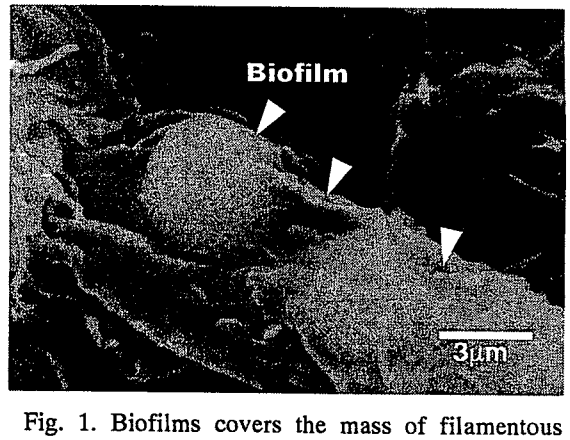

Fig. 1. Biofilms covers the mass of filamentous bacteria and oil droplets (arrows)

cell wall, suggesting the ability to use oil as a source of energy by degrading enzyme in periplasm space. SEM, TEM observation of ultra thin-section and STEM-EDS analyses showed that oil droplets covered with biofilms were nucleated to form covellite (Fig. 2, 3). FT-IR analysis and quantitative analysis of phospholipid indicate the presence of lecithin and phosphoric ester which have a surface-active ability in bacterial EPS and cell wall. The bacterial amphipathic ability led to the easy elemental diffusion through the bacterial cell, therefore resulting in the accumulation of Cu-S by the bacterial cell wall which was bounded by phosphoric ester and N-H.

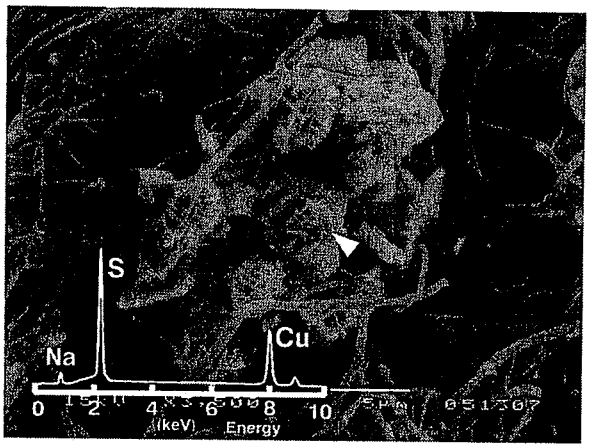

Fig. 2. Covellite mineralization has completed and covellite is formed on the surface of oil droplets, is wrapped in biofilms. The crystalline covellite is composed of high S and Cu with trace of Na (an arrow).

The bacterial amphipathic ability and the specific environment under nearly neutral and anaerobic condition in the hot spring water have a great role in the formation of covellite, which is an effective remediation of petroleum hydrocarbons at oil-polluted area and at Cu-contaminated area.

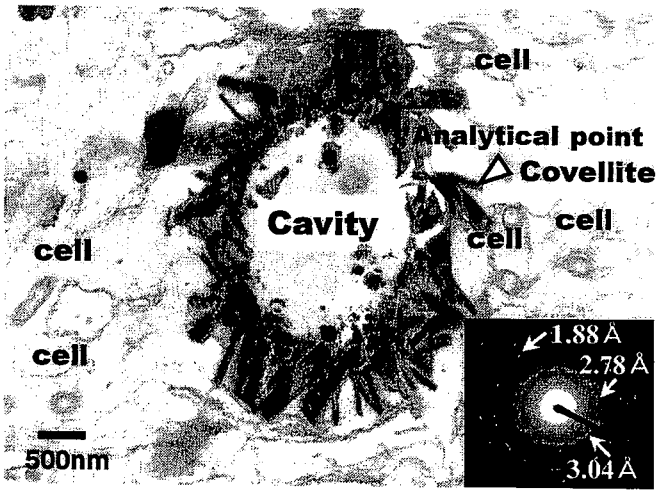

Fig. 3. Crystallized covellite is observed around the cavity of oil droplets. Covellite is surrounded by numerous bacterial cells. Electron diffraction pattern with diffraction spots and rings at 3.04, 2.78 and 1.88 Å (white arrows) indicated at the covellite is highly crystallized mineral.

Biodegradation of crude oil in Niigata oil fields area

GC, GC-MS and GC-MS/MS analyses determined the crude oil degradation level concerned with oil degrading bacteria in Tsukioka hot springs. The susceptibility of oil to microbial degradation is in the following order: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes with lower concentration of normal alkanes, with unresolved complex mixture (UCM). The increasing relative quantities of C_{30} hopane and the absence of pristine, phytane and trepan indicate the evaporation, thus leading to biodegradation. Moreover,

diasteran and regular sterane ratio, Ts/Tm and oleanane/C_{30} hopane ratio also indicate that heavily biodegradation of crude oil, collected from Tsukioka hot springs, has taken place.

Bioremediation of crude oil by diatom in Toyotomi hot springs

Electron microscopical observations revealed crude oil accumulation of diatoms in microbial mats at the Toyotomi hot springs. Crude oil contains Fe, S, Si, and Ca emitting a yellow fluorescence light under the ultraviolet-ray. Microbial mats and hot spring water contain several ions, and water conditions indicate nearly neutral (pH 7.7 and 6.4), anaerobic condition (Eh 37 mV and –14 mV). Optical and epifluorescence microscopic observations show two typed oil fixations; *Pinnularia* spp. and *Frustulia rabenhorst* are encrusted with oil on the surface of cells, whereas *Achnanthes* sp., *Navicula* sp. and/or *Gomphonema* sp. accumulate oil internal cells (Fig. 4). Moreover coccus typed bacteria exist on the surface of oil droplets associated with spherical shaped and filamentous algal, iron oxidation bacteria as a *Leptothrix* sp., and filamentous bacteria. The diatom formed abundant pores (200 nm in diameter) on the cell walls where the oil droplets were filled. The observation still remains to be further analyzed as to the role of the pores not only substance circulation but also oil fixation, and whether such pores are recognized in diatoms.

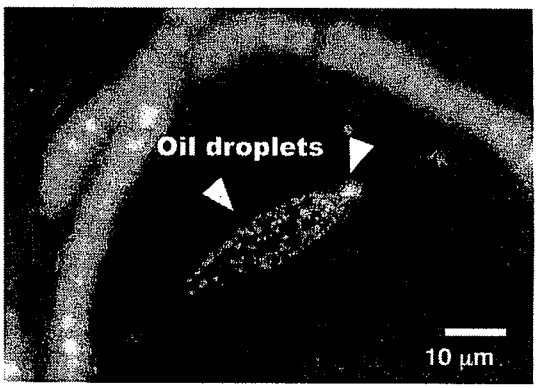

Fig. 4. Diatom cell is about 10 ~ 20 mm in size with accumulation of oil inside the cell (arrows).

The oil fixation of diatom in the oily hot spring water confirms that crude oil is degraded as nutrients and energy source for diatoms. This biodegradation of oil droplets suggests that diatoms have the ability to remediate petroleum hydrocarbons in an oil-polluted area.

Conclusions

Biomineralization of covellite (CuS) and biodegradation of crude oil at oily hot springs, in Japan were studied. In case of Tsukioka hot springs, Cu ion included crude oil, filamentous bacterial degraded metabolism occurred to the Cu-S mineralization depend on the metal species and surrounded environments. In case of iron rich environments, Toyotomi hot springs, iron oxide minerals were formed lead to iron bacterial metabolisms associate with diatom's oil accumulation. The results indicate that the oil degradation microorganisms have an ability of oil degradation associated with heavy metal fixation.

The biomineralization of covellite and the biodegradation of crude oil by filamentous bacteria and diatoms in this study could have profound implications for the bioremediation of not only oil-contaminated site but also heavy metal-polluted area. Thereby, these results provide a significant insight into how bioremediation of the crude oil occurs in hot spring water and how heavy metals influence that bioremediation, thereby giving more fruitful information for the bioremediation of the oil and heavy metals-contaminated environments.

学位論文審査結果の要旨

本学位論文に関し、平成18年2月3日に第1回審査会議を開催、面接審査を行った際、論文の内容について討論した。さらに、2月6日に行われた口頭発表の後に第2回審査会を開き、協議の結果、以下のように判定した。

本論文は原油混じりの温泉水中の生体鉱物化作用および石油を分解する微生物について研究を行った。特に、新潟県月岡温泉水中で銅藍（covellite;CuS）が形成される事を明らかにした。この銅藍の形成には温泉水中に生息する糸状の石油分解菌および球状の硫酸還元菌が直接関与することを各種電子顕微鏡観察で証明した。糸状細菌は二重膜および細胞外多糖類（EPS）を持ち、かつ、界面活性作用を示すレシチンおよびリン酸エステルを含んでいる。また、糸状細菌による油滴中のn-alkanesやisoprenoidの分解をはじめ、hopaneの生成も認められた。バイオフィルムに含まれる有機界面物質により油滴中のCuは温泉水中に溶出し、糸状細菌および球菌の相互作用によって銅藍が形成される。すなわち、石油分解菌の細胞膜およびEPSの有機界面活性作用と硫酸還元菌による水質の還元化が、油滴表面での銅藍の形成を促進する。本論文で明らかにした微生物による石油分解の過程で生じる生体鉱物化作用は、重油およびCuなどの重金属汚染地域における環境浄化にとって有益なデータを提供する。以上の研究成果は博士（理学）に値するものと判定した。