Effect of Kaolinite on Microbial Growth in High Concentration of Heavy Oil

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EFFECT OF KAOLINITE ON MICROBIAL GROWTH IN HIGH CONCENTRATION OF HEAVY OIL

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ABSTRACT

The effect of kaolinite on the growth of hydrocarbon-utilizing indigenous microorganisms in high concentration of heavy oil (i.e., approximately 150 g/l) was studied. Microorganisms were able to grow well in such an extremely high concentration in the presence of yeast extract as co-substrate in direct contact with kaolinite. The presence of kaolinite was not toxic but stimulant for the microbial growth associated with the formation of biofilms at pH values of 5~6 after 36 days of incubation. Bacterial growth predominated when the pH of solutions was neutral-alkaline condition, while fungal growth was predominant as the pH of solutions decreased to be as low as 5 or lower. TEM observation of the kaolinite-oil-microorganism complexes showed that the microbial cells tended to be primarily bound on the edges of kaolinite, and XRD analysis confirmed that their complexes were the adsorption of the cells and heavy oil to the external surfaces of kaolinite. The results may contribute to what environmental factors having a great influence on the bioremediation process is therefore fundamental to many areas contaminated with oil spills, primarily marine and coastal environments. To our knowledge, there are no previous reports on the use of the clay minerals (i.e., kaolinite) in the bioremediation of the *Nakhodka* oil spill in combination with biofilm formation.

Key words: clay minerals, kaolinite, hydrocarbon-utilizing indigenous microorganisms, heavy oil, bioremediation, the *Nakhodka* oil spill

INTRODUCTION

Clay minerals are being used in many applications and there is an increase in their utilization for natural mineral sorbents due to their profusion and economical cost. One of their applications is as an adsorbent or carrier for cleaning up liquid spills (e.g., oil spills). Numerous studies have demonstrated that clay minerals could be employed for the sorption of organic and inorganic compounds (Boyd et al., 1988; Lee et al., 1990; Lin and Puls, 2000). Nevertheless, there is no report about their simultaneous use as adsorbent and carrier in degrading heavy oil. As well, none of the foregoing studies have expressly focused on the mechanisms describing the interactions among clay minerals, heavy oil, and microorganisms.

It has been well known that it is most likely for microorganisms to grow at low substrate concentration. However, not all microorganisms are able to grow on, and consume, high substrate concentration (e.g., heavy oil) as the carbon and energy source, since high substrate concentrations may cause inhibitions in some bacterial enzymatic reactions, which are known as substrate inhibition (Shuler and Kargi, 1992). Hence, our group has established the research addressing the interaction between clay minerals and microorganisms in high concentration of heavy oil (i.e., approximately 150 g/l) (Tazaki, 2003).

The purpose of this study was to elucidate the interaction between clay minerals and hydrocarbon-utilizing indigenous microorganisms in high concentration of heavy oil (collected from the *Nakhodka* oil spill) studied by X-ray powder diffractrometry (XRD), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) in an attempt to determine where and how cell is bound on clay minerals (i.e., kaolinite) that are abundant in nature. In addition, it has been well known that kaolinite is nonswelling 1 : 1 clays, whose binding surfaces are primarily external, and one of the most studied clay minerals, with one layer of tetrahedral stacked on top of an octahedral sheet. It is also the most acidic and temperature-stable clay mineral

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(Li, 2003). On the other hand, it has been known that bonds between oil and smectite results in a loss of expandability of smectite, which ultimately alters the characteristic of smectite to become similar to kaolinite (as nonexpanding clays). Hence, kaolinite was used for this study rather than smectite to investigate whether such nature and behavior of kaolinite were still effective for supporting microbial growth in the uptake of hydrocarbons in conjunction with bioremediation. In addition, the amount (1500 mg/l) of kaolinite was selected for this study with some reasons: (1) the previous research on the use of kaolinite by our group has demonstrated that kaolinite at concentration of 500~2000 mg/l tends to support the growth of hydrocarbon degrading bacteria indigenous to the *Nakhodka* oil spill, whereas kaolinite at concentrations more than 2000 mg/l tends to inhibit bacterial growth (manuscript under preparation); (2) the marine and coastal environment is generally composed of very few clay minerals or low concentration of clay minerals.

MATERIALS AND METHODS

Kaolinite

Kaolinite standard JCSS-1101 (from Kanpaku, Japan) used in this study was obtained from The Clay Science Society of Japan (Okayama, Japan). Kaolinite composed of tubular halloysite and hexagonal kaolinite (based on the TEM observation) was used in batch experiments without any pretreatment.

Bacterial strain

Apart from microorganisms inhabiting the natural seawater (NSW) and heavy oil, the bacterium capable of degrading heavy oil was also added as inoculum in order to promote the degradation of heavy oil. The bacterium was isolated from Atake seashore, Ishikawa Prefecture, Japan. Using 16S rDNA sequencing, this bacterium was affiliated to *Pseudomonas aeruginosa* (98% similarity). This bacterium was selected for study because it produced high extracellular emulsifying activity after 2 days of incubation in the presence of heavy oil (2% v/v) and 1 g/l of yeast extract (Chaerun et al., 2003).

Bacterial culture and batch experimental conditions

Batch bacterial experiments were conducted with kaolinite standard JCSS-1101 (from Kanpaku, Japan). The growth medium contained the necessary components needed for bacterial growth. The following were included for each liter of medium: 25 g of NH_4NO_3, 0.5 g of $FeC_6H_5O_7 \cdot nH_2O$ (ferric citrate), and 0.5 g of K_2HPO_4 per liter distilled, deionized water (NDW). Natural seawater (NSW) without filtration (collected from the Sea of Japan) was also added to each flask as additional medium. Briefly, the batch reaction vessels consisted of acid-washed 1000 ml Erlenmeyer flasks containing 600 ml of growth medium: 480 ml of NSW was mixed with 120 ml of an autoclaved NDW. The kaolinite (1500 mg/l) was added to the flask and the pH of the medium was adjusted to pH 7.8 with 1N NaOH solution. Immediately after sample preparation, before introducing the inocula originating from the heavy oil and NSW medium into the flasks, all the three flasks with their contents were autoclaved, allowed to cool to room temperature (24°C), and inoculated with NSW medium, the stock culture of bacterial strain *Pseudomonas aeruginosa* (4.5% v/v), and heavy oil (collected from the *Nakhodka* oil spill) to a final concentration of approximately 150 g/l, serving as the sole carbon and energy sources (Chaerun and Tazaki, 2003; Chaerun et al., 2003; Tazaki, 2003).

Overall, three experimental flasks were established: one main flask contained medium, microorganisms, and kaolinite (coded as R1); one abiotic control flask contained medium and kaolinite (coded as R2); and one biotic control flask contained medium and microorganisms (coded as R3). To avoid microbial growth and ensure sterility during the experiment, abiotic control flask was autoclaved three times and heavy oil solution was sterilized by filtration through a 0.22 μm pore-sized filter. Cultures were incubated for 105 days at room temperature (24°C) with shaking at 125 rpm and allowed microorganisms to grow. Samples were removed periodically at sampling periods of 0, 36, 64, and 105 days. Separate sets of samples were made up and prepared for analyses by X-ray diffraction analysis (XRD), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). In addition, the liquid medium of two experimental systems (i.e., R1 and R3) was supplemented with 1 g/l of yeast extract after 36 days of the course of the experiments.

X-Ray Diffractometery (XRD)

Relative changes in the basal spacings of kaolinite were measured by X-ray powder diffraction (XRD). Samples were prepared by spreading 2 mg of the suspended clays over a 2.5-cm^2 area of a glass slide. The slides were air-dried, placed in a desiccator containing silica gel to prevent rehydration, and observed using CuKα generated at 40 kV and 30 mA by using the $2\theta/\theta$ method and a scan speed 2°/min (Rigaku Rinto 1200 X-ray diffractometer).

Microscopic techniques

For SEM observation, freeze-drying was used for sample preparation (Suzuki et al., 1995). Briefly, the suspension samples were fixed with 2.5% (vol/vol) glutaraldehyde, pipette-drawn, mounted on 0.22 μm membrane filter, washed and fixed with t-butyl alcohol, subsequently frozen in liquid nitrogen, and dried up with low-vacuum SEM. After freeze-drying completely, samples were transferred to the brass-stub with double-sided adhesive carbon tape, coated with carbon, and then observed by using a scanning electron microscope (JEOL JSM-5200 LV). In case of TEM observation, samples were fixed with 2.5% v/v glutaraldehyde for 2 hours at 4°C, mounted on copper specimen grids, allowed to dehydrate at room temperature, and then viewed using a

JEOL JEM-2000EX transmission electron microscope.

Analytical techniques

The pH in solution was monitored using a pH meter (Horiba) at set time intervals. For enumeration of total cells in the biofilm and planktonic milieu, 1 ml of the homogenized solution (containing both free-living cells and cells that were attached to kaolinite surfaces) was taken into vials, sonicated at 4°C for 3 min, vortexed for 10 seconds to disperse the microorganisms attached to kaolinite surfaces. The resuspended biofilm or plantonic sample was serially diluted with fresh minimal salts media, and plated in nutrient agar (NA) plates supplemented with 1 g/l yeast extract and 0.85% (w/v) NaCl, then incubated at 25°C for 3 to 5 days. For statistics, analyses of samples were performed in triplicate. The data are presented as the arithmetic means ± standard deviations of the mean.

RESULTS

Microbial growth in experimental systems

During the first 36 days, microbial growth appeared to be a lag phase. That is, there was no significant increase in microbial cell number of all experimental systems (Fig. 1). After 36 days of incubation, 1 g/l of yeast extract was introduced to two flasks (R1 and R3). The supplement of yeast extract resulted in a sharp increase in microbial cell number in R1 but not in R3, indicating that only in the presence of kaolinite, cells had adjusted to the solution with high concentration of heavy oil and were able to multiply sharply resulting in an exponential increase in cell mass and cell number density with time. The maximum microbial growth occurred at 64 days in R1. Subsequently, after 64 days of incubation up to the end of experiment (at 105 days), cell numbers appeared to decline markedly. The results showed that the presence of kaolinite in such a high concentration of heavy oil could stimulate microbial growth.

pH and microbial succession

During the first 36 days of incubation, the solution pHs of three experimental systems (R1, R2, and R3) were maintained between 7.7 and 7.8. However, after 36 days of incubation, microbial growth significantly decreased the pH of the solution from 7.8 to 6.3 for R1 and from 7.7 to 3.7 for R3, while abiotic control (R2) maintained the initial pH of 7.5~7.8 over a period of experiment (Fig. 2). Once the pH reached a neutral value in solution of abiotic control, it remained relatively constant suggesting the equilibrium was established between the oil-kaolinite complexes and the solution. At 64 days, the pH values of the solutions of R1, R2, and R3 reached 6.3, 7.5, and 3.7, respectively. At the end of the experiments at 105 days, the pH values of solutions of R1, R2, and R3 were 5, 7.7, and 3.4, respectively.

During the first 36 days of incubation, bacterial growth was predominant in both R1 and R2 ranging from 98.5% to 100%, while fungal growth was very low ranging from 0% to 1.5%. At 64 days, when the solution pH of R1 decreased to 6.3, bacterial growth still predominated (70%) and fungal growth increased up to 30%, while fungal growth predominated (99.5%) and bacterial growth decreased to 0.5% in R3 when the solution pH decreased to be as low as 3.7. At the end of the experiment (at 105 days), fungal growth was predominant in both R1 (99.9%) and R3 (99.5%) when the solution pH decreased to be as low as 3~5 (Table 1).

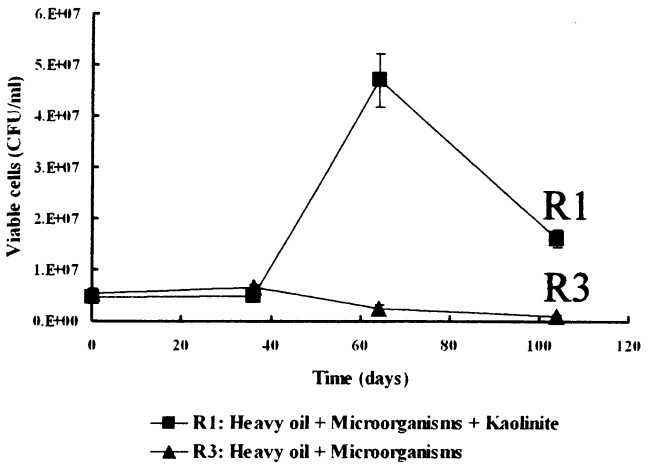

FIG. 1. Effect of kaolinite on microbial growth (bacteria and fungi) as viable cells in colony forming unit (CFU) per ml during the 105-day incubation. Error bars represent standard deviations based on three replicates.

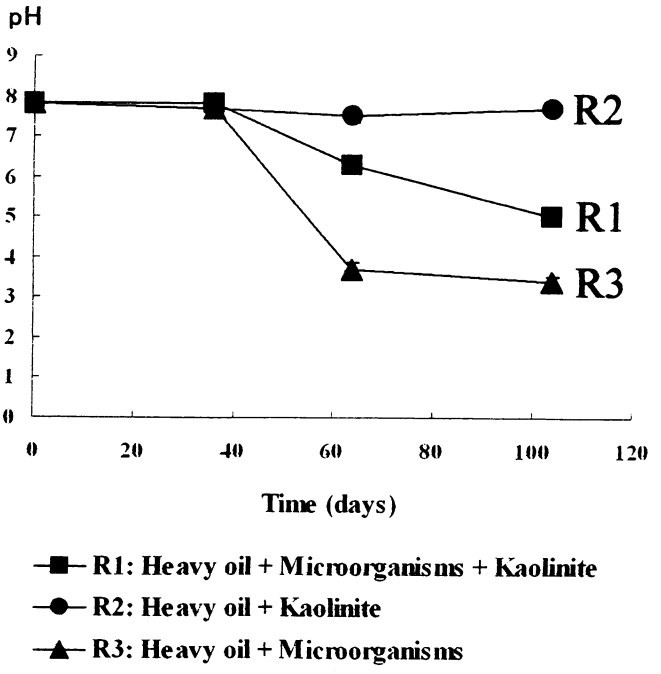

FIG. 2. Effect of kaolinite on pH changes in liquid culture of three experimental systems during the 105-day incubation.

TABLE 1. Microbial succession or the predominant microorganism (bacteria and fungi) during the 105-day incubation.

Experimental system	0 day	36 days	64 days	105 days
R1: heavy oil + microorganisms + kaolinite	bacteria (100%) fungi (0%)	bacteria (99.5%) fungi (0.5%)	bacteria (70%) fungi (30%)	bacteria (0.5%) fungi (99.5%)
R3: heavy oil + microorganisms	bacteria (100%) fungi (0%)	bacteria (98.5%) fungi (1.5%)	bacteria (0.5%) fungi (99.5%)	bacteria (0.5%) fungi (99.5%)

X-Ray Diffractometery (XRD)

Figure 3 represents the basal spacings of kaolinite (7.1 Å) and of its complexes with microbial cell and/or heavy oil. Both kaolinite-oil and kaolinite-oil-cell complexes did not substantially lead to an increase in the basal spacing of kaolinite. The binding and adsorption of coincident heavy oil and cells did not bring about an increase in the basal spacing of kaolinite and no expansion of kaolinite was observed, as kaolinite was nonexpanding clays (Stotzky, 1986), indicating that heavy oil and cells did not intercalate kaolinite. In addition, kaolinite is nonswelling 1:1 clays, whose binding surfaces are primarily external, so that no significant increase of basal spacing was observed throughout the experiments. However, the intensity of the basal spacings of kaolinite (7.1 Å) increased constantly throughout the experiments in both R1 and R2, suggesting that the microbial degradation of heavy oil occurred in R1, and the adsorption of heavy oil by kaolinite occurred in R2.

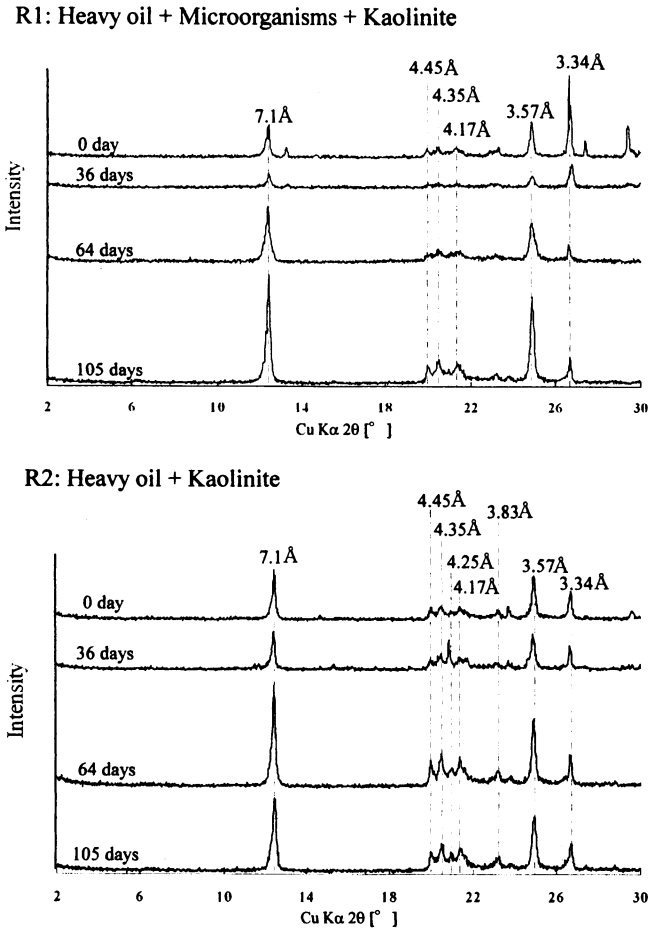

FIG. 3. X-ray powder diffraction analyses of heavy oil + microorganisms + kaolinite (R1) and heavy oil + kaolinite (R2). Note that the intensity of the 7.1 Å peak increases with the incubation times.

Scanning electron microscopy

Figure 4 presents the images of a dense network of cells and several clusters of kaolinite (R1) after 64 days of the course of the experiment. The tubular halloysite, fungal hyphae, and bacterial cells appeared to connect with one another (Fig. 4A). The structure of their connection appeared as somewhat roughened matrix where tubular halloysite had the size of approximately 1–1.3 μm in width. The interactions among kaolinite, cells, and heavy oil resulted in several dense agglomerations as the kaolinite-cell complexes consisting of microbial bodies (bacterial cells), flagella (fungal hyphae and spores), fibrous attachment feature, and kaolinite particles embedded in more amorphous-looking organic extracellular exudate (Fig. 4B). These configurations could be visually observed by scanning electron microscopy where cells and fibrous attachment features were clearly visible. Kaolinite particles tended to have somewhat roughened matrix with relatively rough surface microtopography. Most of the cells tended to emerge outside the biofilm matrix so that the cells were easy to discern on kaolinite. Cells appeared to develop on kaolinite and thrive in connection with kaolinite particles as biofilm matrix (Fig. 4B). The sizes of fungal hyphae were in the range of 0.4–0.5 μm in width, while the bacterial cell sizes were in the range of 0.2–0.4 μm in width and 0.8–1.4 μm in length.

Transmission electron microscopy

TEM images (Figs. 5 and 6) more clearly showed the binding and adsorption of the cells on kaolinite than did SEM (Fig. 4). The cells appeared to be predominantly bound on the edges of tubular halloysite and hexagonal kaolinite (Figs. 5B, 6A, and 6B). Kaolinite displayed short and long tube-like shape (tubular halloysite) and hexagonal shape (Fig. 5A). At the beginning of the experiment and after 36 days of incubation in experimental system R1, tubular halloysite, hexagonal kaolinite, and bacterial cells appeared as adsorptive or porous materials that were surrounded by the sticky oily thin films and/or extracellular polymeric substances (EPS) (Figs. 5A and 5B). In contrast, bacterial cells, hexagonal kaolinite, and tubular halloysite with high dense as well as a small amount of oily thin films and/or

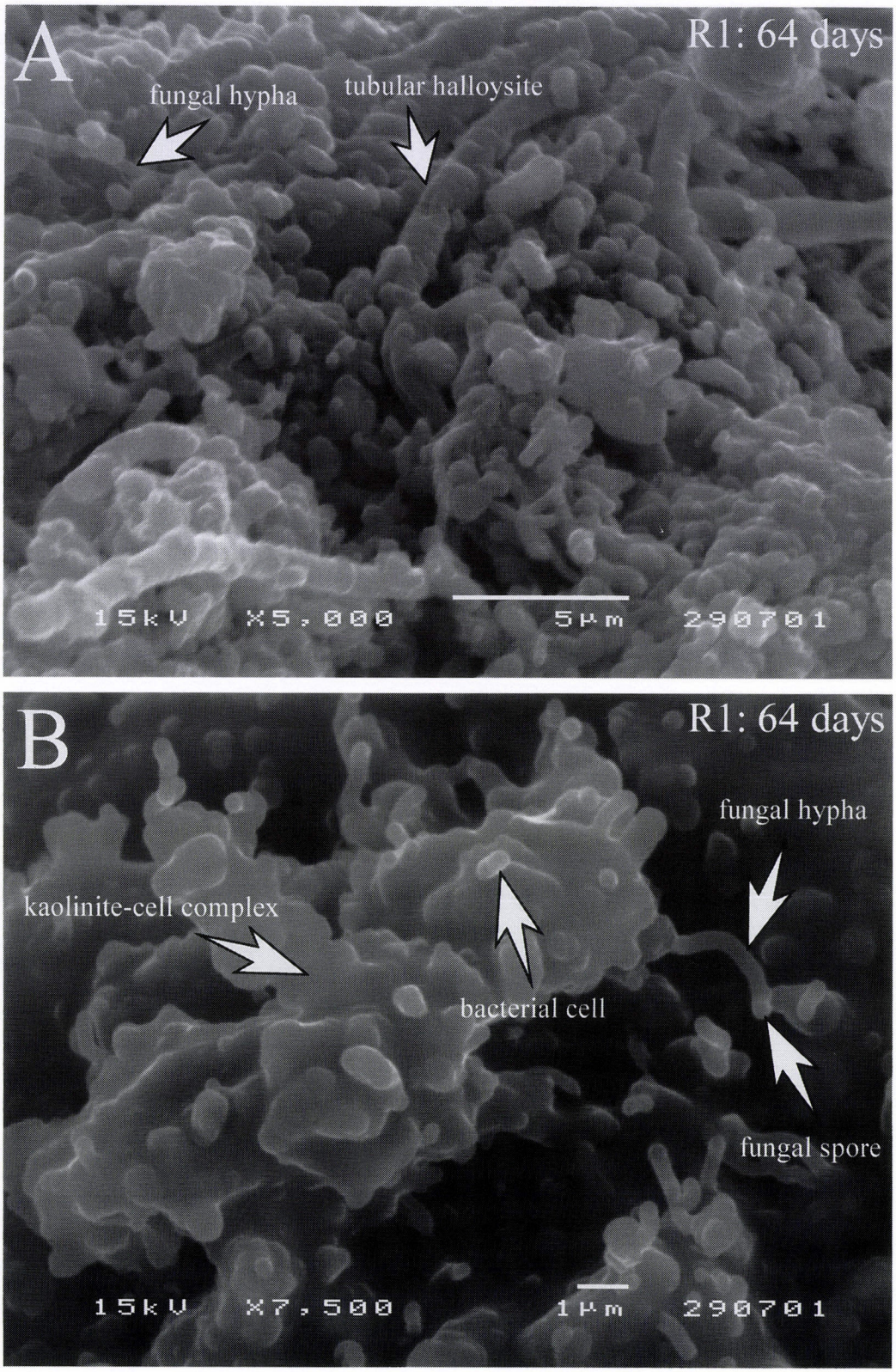

FIG. 4. SEM images of microbial cells associated with biofilm formation on kaolinite in R1 after 64 days of incubation showing the feature of tubular halloysite and hexagonal kaolinite aggregated with microbial cells (A), and the development of fungal-bacterial cells on kaolinite as biofilm matrix (B).

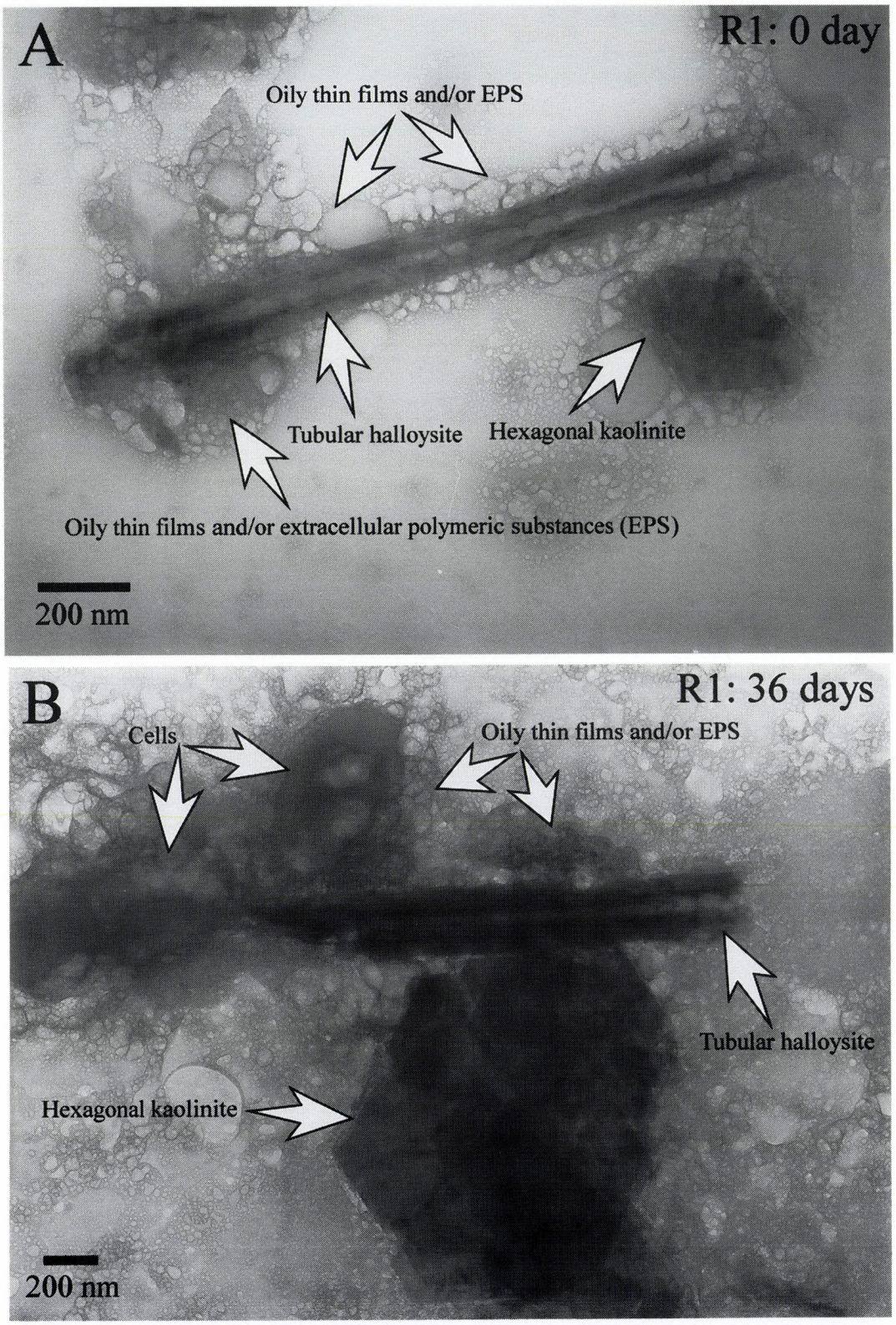

FIG. 5. TEM images of kaolinite at the onset of the experiment in R1 showing two types of kaolinite crystals (tube and hexagonal plates) (A), and cells bound on the edges of tubular halloysite in connection with hexagonal kaolinite after 36 days of incubation (B).

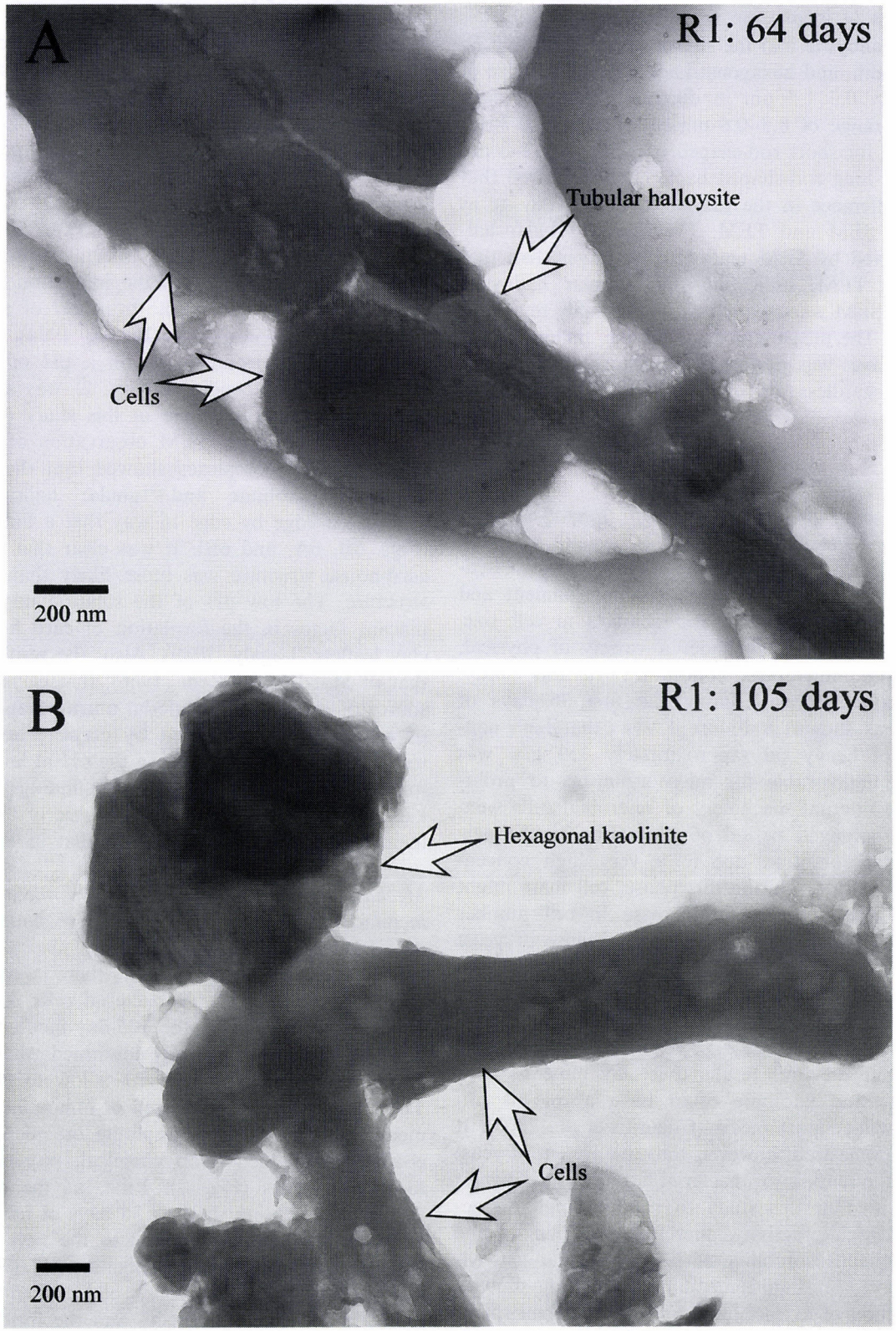

FIG. 6. TEM images of short-rod shaped cells bound on tubular halloysite showing the high dense bacterial cell and tubular halloysite after 64 days of incubation (A), and high dense long-rod shaped cells bound on high dense hexagonal kaolinite after 105 days of incubation (B).

EPS were observed in experimental system R1 after 64 and 105 days of incubation (Figs. 6A and 6B).

Tubular halloysite had the size of approximately 0.1–0.3 μm in width, and hexagonal kaolinite had the size of approximately 0.3–1.5 μm in diagonal. Bacterial sizes were in the range of 0.3–0.6 μm in width and 0.7–1.2 μm in length for short rod-shaped bacteria, and 2–3 μm in length for long rod-shaped bacteria. It appeared that there was difference in the size of tubular halloysite as observed by SEM and TEM. The size of tubular halloysite observed by SEM tended to be larger than that observed by TEM, indicating that tubular halloysite observed by SEM was covered with sticky oily thin films and/or EPS. The measurement of tubular halloysite size for TEM images was performed on the original tubular halloysite, regardless of oily thin films and/or EPS around it.

DISCUSSION

Effect of kaolinite on microbial growth, microbial succession, and the pH of solution

For microorganisms, growth is their most essential response to their physiochemical environment and a result of both replication and changes in cell size. Microorganisms can grow under a variety of physical, chemical, and nutritional conditions. In this study, the lag phase occurring during the first 36 days of incubation, as shown in Figure 1, was that high concentration of heavy oil (approximately 150 g/l) was presumably unfavorable for microorganisms to proliferate or the internal machinery of microbial cells went through a prolonged period of adaptation to the new environmental condition due to a very high concentration of substrate. During this phase, cell mass might increase a little, without an increase in cell number density. It was also possible that the absence of yeast extract as co-substrate had a strong effect on the length of lag phase. Inversely, no increase in cell number in R3 was a result of fungal-bacterial competition in which fungi predominated (Table 1). The results obtained here confirmed our previous results that clay minerals such as bentonite and kaolinite could have a role as microbial growth-support media (Chaerun et al., 2003). It should be emphasized, however, that the present of yeast extract was essential for bacterial strain *Pseudomonas aeruginosa* used in this study to produce high extracellular emulsifying activity, therefore resulting in the apparent aqueous solubility of heavy oil that was visually observed in liquid medium (figure not shown). The production of extracellular emulsifying agent might be associated with hydrocarbon utilization. Emulsification facilitated solubilization of hydrocarbons by increasing their surface area. Solubilization might facilitate hydrocarbon uptake into microbial cells where it could be metabolized if appropriate enzymes were present (Berg et al., 1990). Once a hydrocarbon was emulsified, it might be easier to solubilize by increasing the surface area of the hydrocarbon (Cooper, 1986).

Correspondingly, a substantial decrease in the solution pH of R1 and R3 was strongly affected by microbial growth, since an abiotic control (R2) did not have an impact on the solution chemistry (Fig. 2). Presumably, both cellular metabolic activity and the resulting degradation product of heavy oil all contributed as driving forces for this chemical parameter. As reported by other researchers that microbial growth generally results in a decrease of the pH of the medium by release of acidic metabolites (e.g., organic acids, H_2SO_4). The pH, thereafter, affects the activity of microbial enzymes as well as the ionization of chemicals and thus plays a role in the transport of nutrients and toxic chemicals into cell (Bitton, 1994). Since fungi prefer acidic environments, with a pH of 5 or lower, such a chemical condition (Fig. 2) was extremely favorable for fungi to thrive in this study (Table 1 and Fig. 4). Furthermore, TEM observation of the clay-oil-microorganism complexes showed that the particles of hexagonal kaolinite and tubular halloysite mostly aggregated edge by edge in way that a linked structure (Figs. 5B, 6A, and 6B). It was clear that the resulting card-house structure was more likely than a band-like structure. The low pH of the solution might be a significant factor in the formation of card houses by the clay leaflets (Lagaly, 1989). Also, the results confirmed that of Stotzky and Rem (1966), that clays served as a source of minerals for bacterial nutrition and respiration of bacteria was stimulated by clays, primarily as the result of the clays maintaining the pH at levels adequate for sustained growth. Our present findings showed that kaolinite in R1 could stimulate the microbial growth and maintain the pH compared with that in R3 (Fig. 2).

Mechanisms of effect of kaolinite on microbial growth in relation to the formation of the kaolinite-oil-cell complexes

TEM images showed that there were significant changes in the feature of bacterial cells, kaolinite, and halloysite in R1 during the 105-day incubation (Figs. 5 and 6). XRD data also confirmed that crystalline kaolinite changed gradually from low to high (Fig. 3). Their changes are summarized in Figure 7. The possible mechanisms of effect of kaolinite on microbial growth could be explained upon chemical, physical, and biological processes (Fig. 7). First, at the onset of the experiments (during the first 36 days of incubation), the adaptation of microorganisms to the new environment under conditions of neutral-alkaline pH (7.8 ~ 8.0) and high concentration of heavy oil resulted in little increase in cell number density and the formation of the kaolinite-oil-cell complexes that were surrounded by sticky oily thin films and/or extracellular polymeric substances (often exopolysaccharides) where hexagonal kaolinite and tubular halloysite were porous materials. Second, after the cells had adjusted to their new environment (for another 70 days of incubation), cells consuming heavy oil were able to multiply rapidly, and cell number density increased exponentially with time, resulting in

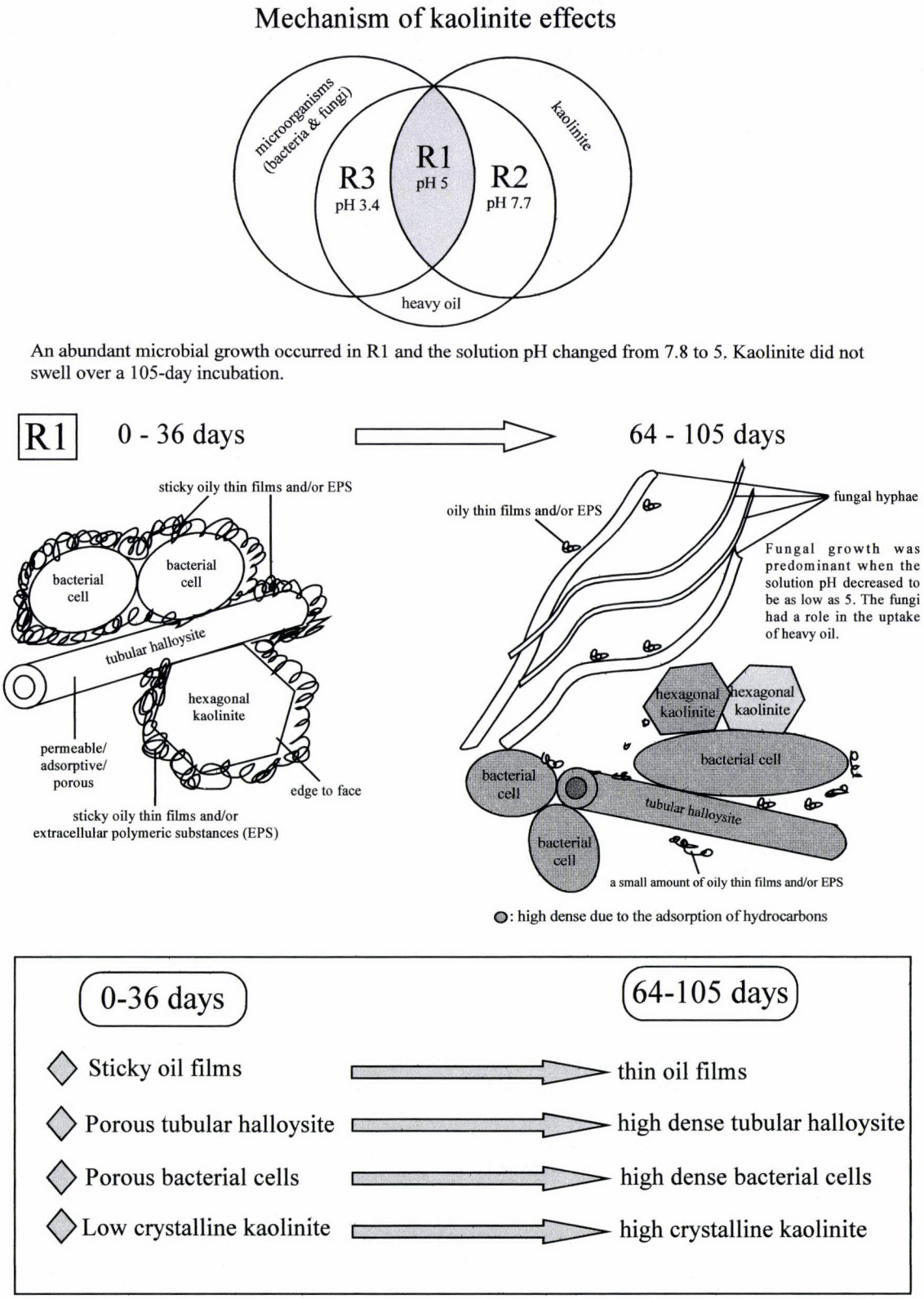

FIG. 7. Schematic diagram of mechanisms of effect of kaolinite on microbial growth, microbial succession, and the solution pH showing nonswelling-kaolinite, as well as the formation of the kaolinite-oil-cell complexes showing changes in the structure and feature of bacterial cells, tubular halloysite, and hexagonal kaolinite from porous materials to high dense materials due to the adsorption of hydrocarbons during the 105-day incubation in high concentration of heavy oil.

lowering the pH (as low as 5) as well as allowing fungi to thrive. Last, the uptake and/or the adsorption of heavy oil by cells, hexagonal kaolinite, and tubular halloysite resulted in a small amount of oily thin films surrounding the kaolinite-oil-cell complexes, as indicated by high denseness of cell, hexagonal kaolinite, and tubular halloysite.

Correspondingly, the low pH of the aqueous solution in R1 (pH 5) resulted in an acid-treated kaolinite that might be effective catalysts for the biological reactions such as microbial growth. The formation of biofilms occurring as the kaolinite-oil-cell complexes in R1 might also act as metabolically active interfaces between bulk fluid and solid mineral phases, therefore having a profound effect on greater microbial growth than that in nonkaolinite-containing biotic control (R3). The catalytic activity of kaolinite for microbial growth in high concentration of heavy oil in this study might be important for the heavy oil-polluted areas in relation to bioremediation.

CONCLUSION

During the period of 36–105 days, the presence of kaolinite allowed microorganisms to grow very well above levels of non-kaolinite-containing biotic control. The maximum microbial growth occurred at 64 days reaching 4.7×10^7 CFU/ml in cell number. There were two kinds of the predominant microorganisms, that is, bacteria and fungi. Bacterial growth was predominant under neutral-alkaline conditions (pH 6.3∼7.8), while fungal growth was predominant when the solution pH decreased to be as low as 5 or lower. In addition, the basal spacing (7.1 Å) of kaolinite did not change throughout the experiments.

Our present results also provided evidence that when microorganisms were associated with naturally occurring inorganic particulates such as kaolinite in the oil spill-contaminated marine and coastal environments, kaolinite would be able to stimulate microbial growth in combination with biofilm formation. It was suggested that kaolinite might serve as catalyst other than the microbial growth-supporting media in the proliferation of microbial cells in high concentration of heavy oil. The results might have a profound effect on the bioremediation of the oil spill-polluted sites, especially marine and coastal environments.

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REFERENCES

Berg, G., Seech, A.G., Lee, H. and Trevors, J.T. (1990) Identification and characterization of a soil bacterium with extracellular emulsifying activity. *J. Environ. Sci. Health*, **A25 (7)**, 753–764.

Bitton, G. (1994) Wastewater microbiology. John Wiley & Sons, New York.

Boyd, S.A., Shaobai, S., Lee, J. and Mortland, M.M. (1988) Pentachlorophenol sorption by organo-clays. *Clays Clay Miner.*, **36**, 125–130.

Chaerun, S.K. and Tazaki, K. (2003) Hydrocarbon-degrading bacteria in the heavy oil polluted soil and seawater after 5 years of bioremediation, p. 187–204. *In* K. Tazaki (ed.), Water and Soil Environments: Microorganisms play an important role. 21st century COE Kanazawa University, Kanazawa University Press, Kanazawa, Japan.

Chaerun, S.K., Tazaki, K. and Asada, R. (2003) Double function of bentonite and kaolinite as adsorbents and "microbial growth-support media" for degradation of crude oil, p. 253–277. *In* Tazaki, K. (ed.), Heavy Oil Spilled from Russian Tanker "Nakhodka" in 1997: Towards Eco-responsibility, Earth Sense. 21st Century COE Kanazawa University, Kanazawa University Press, Kanazawa, Japan.

Cooper, D.G. (1986) Biosurfactant. *Microbiol. Sci.*, **3**, 145–149.

Lagaly, G. (1989) Principles of flow of kaolin and bentonite dispersions. *Appl. Clay Sci.*, **4**, 105–123.

Lee, J., Mortland, M.M., Chiou, C.T., Kile, D.E. and Boyd, S.A. (1990) Adsorption of benzene, toluene, and xylene by two tetramethylammonium-smectites having different charge densities. *Clays Clay Miner.*, **38**, 113–120.

Li, L.Y. (2003) Multi-component of heavy metal contaminants adsorptivity and compatibility onto variable charge clay mineral. *Clay Science*, **12**, 73–80.

Lin, Z. and Puls, R.W. (2000) Adsorption, desorption and oxidation of arsenic affected by clay minerals and aging process. *Environ. Geol.*, **39**, 753–759.

Shuler, M.L. and Kargi, F. (1992) Bioprocess engineering basic concepts. Prentice Hall International, New Jersey, pp. 61–78.

Stotzky, G. and Rem, L.T. (1966) Influence of clay minerals on microorganisms. I. Montmorillonite and kaolinite on bacteria. *Can. J. Microbiol.*, **12**, 547–563.

Stotzky, G. (1986) Influence of soil minerals colloids on metabolic processes, growth, adhesion, and ecology of microbes and viruses, p. 305–428. *In* P.M. Huang and M. Schnitzer (eds.), Interactions of Soil Minerals with Natural Organics and Microbes. Soil Science Society of America, Madison.

Suzuki, T., Shibata, M., Tanaka, K., Tsuchida, K. and Toda, T. (1995) A new drying method: low vacuum SEM freeze drying and its application to plankton observation. *Contribution to the Bulletin of Planktonic Society of Japan*, **42**, 53–62.

Tazaki, K. (ed.). (2003) Heavy oil spilled from Russian tanker "Nakhodka" in 1997: towards eco-responsibility, earth sense, 21st Century COE Kanazawa University, Kanazawa University Press, Kanazawa, Japan.