Double function of bentonite and kaolinite as adsorbents and "Microbial growth-support media" for degradation of crude oil

DOUBLE FUNCTION OF BENTONITE AND KAOLINITE AS ADSORBENTS AND "MICROBIAL GROWTH-SUPPORT MEDIA" FOR DEGRADATION OF CRUDE OIL

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ABSTRACT

The effects of bentonite and kaolinite as adsorbents and microbial growth-support media in enhancing the degradation of crude oil were investigated. Kaolinite was a more effective adsorbent for the removal of crude oil than bentonite. Both clays had an essential role as microbial growth-support media in accelerating the degradation of crude oil, which was influenced by a biofilm formed on their surfaces. The best biofilm formation as shown by the thickness of the biofilm occurred on bentonite compared with on kaolinite, which was shown by increasing the thickness of biofilm on its surface. XRD analysis showed that the basal spacings of bentonite and kaolinite were not altered, indicating that the crude oil and microbial cells did not significantly intercalate the clays. However, total count of basal spacing declined constantly throughout the experiments for kaolinite in the presence and absence of microorganisms, but with bentonite in the presence of microorganisms, suggesting that microorganisms were mainly responsible for disordering the clays. Additionally, FT-IR analyses suggested that the structure of bentonite and

kaolinite, such as OH bonding, Al-OH bonding, and Si-O bonding, changed in the presence of both crude oil and microorganisms, indicating that the clays interacted with crude oil and microorganisms not only physically but also chemically. With respect to the extent of their adsorptive potentials for crude oil, FT-IR data demonstrated that microorganisms enhanced the adsorptive capability of bentonite but not of kaolinite. Scanning and transmission electron microscopy showed that the binding of microbial cells was primarily on the edges and planar surfaces of the clays. It was concluded that degradation of crude oil resulted from a synergism between the clay minerals and hydrocarbon-degrading microorganisms.

INTRODUCTION

Adsorption is the collection of a substance onto the surface of an adsorbent solid. Several factors affect adsorption: e.g., temperature, contact time, concentration, and surface area of adsorbent (Cheremisinoff *et al.,* 1978; Stotzky, 1986, Chaerun and Wisjnuprapto, 2000). Clays are employed as adsorbent for both organic (humic) and inorganic (e.g., heavy metals) pollutants (Boyd *et al.,* 1988; Lee *et al.,* 1990; Michot and Pinnavaia, 1991; Kayabali and Kezer, 1998; Lin and Puls, 2000; Cooper *et al.*, 2002). Adsorption of organic pollutants was enhanced by the interaction of aliphatic amines with montmorillonite (Wolfe *et al.,* 1985), and tetramethylphosponium- and tetramethylammonium- smectite were employed as adsorbents of aromatic and chlorinated hydrocarbons (Kukkadapu and Boyd, 1995). Although clay minerals are generally ineffective sorbents for removing organic pollutants from water, their sorptive capabilities for organic contaminants can be enhanced substantially by replacing naturally-occurring inorganic exchange cations with organic cations (Mortland *et al.*, 1986; Smith *et al.*, 1990; Lee *et al.*, 2002).

Degradation of benzylamine was inhibited when incubated in the presence of montmorillonite (Miller and Alexander, 1991; Knaebel *et al.,* 1994), and the degradation of diquat was inhibited in the presence of montmorillonite but not in the presence of kaolinite (Weber and Coble, 1968; Knaebel *et al.,* 1994). Conversely, there were substantial deceases in the rates and extents of degradation of phenol and glutamate in the presence of kaolinite (Scow and Alexander, 1992; Knaebel *et al.,* 1994). Other

studies have shown that biodegradation of pentachlorophenol and toluene was not retarded by interaction with the soil matrix (Bellin *et al.*, 1990; Robinson *et al.*, 1990; Knaebel *et al.*, 1994). Sorption of pentachlorophenol to alkaline and acidic soils increased with increasing additions of sludge, but biodegradation was not affected (Berlin *et al.*, 1990; Knaebel *et al.*, 1994). Sludge additions did not significantly affect either adsorption or biodegradation of 2,4-dichlorophenol in calcareous soils (O'Connor *et al.*, 1990; Knaebel *et al.*, 1994).

In addition to their application in biological system (e.g., biological treatment processes), clays have a double function as adsorbents and microbial growth-support media (Ueshima and Tazaki, 2001). Cells can be bound directly to a support material/medium (i.e., clays), and an immobilization of cells on the surfaces of support materials can be achieved by physical adsorption or covalent binding. The major advantage of immobilization by adsorption is a direct contact between nutrient and support materials, resulting in high cell loading associated with attached-growth of microorganisms (biological films). Biological films or biofilms are the multilayer growth of cells on solid support surfaces where the support material can be inert or biologically active (Chaerun and Wisjnuprapto, 2000).

However, little is known about the simultaneous interactions among organic chemicals, added clay minerals, and microbial activity on the removal or degradation of organic chemicals (e.g., crude oil as a pollutant). The present study is unique in that the simultaneous actions in the adsorption and biodegradation of organic chemical (herein crude oil collected from the *Nakhodka* oil spill) along with biofilm formation were evaluated. Three processes might be involved: physical process by adsorbents (e.g., bentonite and kaolinite), biological process by microorganisms, and a combination of physical and biological processes (clays and microorganisms). Experiments were designed on the role of clay minerals (bentonite and kaolinite) in either adsorbing crude oil or supporting the growth of microorganisms (biofilm formation), on the degradation of crude oil. This study also differentiated the effects of bentonite and kaolinite on overall microbial activity and on the extent of their adsorption potentials. This research, therefore, has a great significance and provides valuable insights into what factors are keys in influencing the bioremediation/degradation of oil spills. To our knowledge, there

are no previous reports on the use of these clays in degradation/bioremediation of the *Nakhodka* oil spill in combination with biofilm formation.

MATERIALS AND METHODS

Clay samples

The clay minerals used were bentonite and kaolinite. Na-bentonite was supplied by Kunimine Company, Japan. Kaolinite (kaolin standard) was obtained from New Zealand.

Crude oil samples

Crude oil sample was collected from the *Nakhodka* oil spill at Atake Seashore, Ishikawa Prefecture, Japan. The *Nakhodka* Russian oil occurred on the 2nd of January 1997, which discharged approximately 6,240 kl of C-heavy oil into the Japan Sea. The heavy oil spill led to a serious impact to the surrounding environment, particularly the heavy oil pollution of the shoreline from Mikuni, Fukui Prefecture to Noto Peninsula, Ishikawa Prefecture (Tazaki et al. 1997a; Tazaki et al. 1997b; Tazaki et al. 1997c; Tazaki 1998). The chemical composition of the drifted heavy oil was mainly aliphatic hydrocarbon, that is, n-alkanes of C_9-C_{30}, in which n-eicosane (n-$C_{20}H_{42}$) is the most abundant compound, while the contents of aromatic compounds were low (Tazaki et al. 1997b; Tazaki et al. 1997c; Shibata et al. 1997; Itagaki and Ishida 1999).

Experimental design

The laboratory experiment was designed in a batch culture by shaking on a rotary shaker (125 rpm). There were six experimental systems, including controls: two experimental systems were for a combination of biological and physical processes, two experimental systems were for physical process only (as abiotic controls), and two experimental systems were for biological process only (as biotic controls). A series of 300-ml Erlenmeyer flasks were used for this experiment. Each flask contained 200 ml of a natural seawater medium (NSW): 160 ml of natural seawater without filtration was mixed with 40 ml of an autoclaved solution containing nitrogen, phosphorous, and iron nutrients (1.0 g of NH_4NO_3, 0.02 g of $FeC_6H_5O_7.nH_2O$, 0.02 g of K_2HPO_4 in 40 ml of distilled water), and 1 g/l of yeast extract as co-substrate. The pH of medium was

adjusted to pH 7.8 with NaOH. Crude oil collected from the crude oil-polluted coastal area at Atake Seashore (Ishikawa Prefecture) in Japan was added to the NSW to a final concentration of 10 mg/ml as the sole carbon and energy source (Chaerun and Tazaki, 2003). Microorganisms inhabiting the NSW and the crude oil were used as inocula. Apart from bacteria inhabiting the NSW and the crude oil, the bacterium capable of degrading crude oil was also added as inoculum in order to promote the degradation of crude oil. The bacterium was isolated from Atake Seashore, Ishikawa Prefecture, Japan. Preliminary experiments have shown that this bacterium produces biosurfactants that enhance the apparent aqueous solubility of crude oil as well as result in increased bioavailability and biodegradation (unpublished data). Cultures were then amended with 1000 mg/l of bentonite and kaolinite, and incubated at room temperature for 15 days. Two each flasks without clay but with either NSW or the enrichment medium and two each flasks without microorganisms but with either bentonite or kaolinite were also incubated as biotic and abiotic controls, respectively. The biotic control with the enrichment medium contained: 1 g/l of crude oil, 8 g/l of nutrient broth, 1 g/l of yeast extract, and 0.85% (w/v) NaCl. Before introducing the inocula that originated in the crude oil and the NSW medium, the six flasks with their content described above were sterilized by autoclaving for 25 min at 121°C, and two uninoculated flasks (abiotic controls) were autoclaved three times to avoid the growth of microorganisms in the crude oil, seawater, and clay constituents, as well as to ensure sterility during the experiment. Samples were removed three times: at the beginning of the incubation on day 0, after 7 days of incubation, and at the end of incubation on day 15. To facilitate description, each experimental system was coded: a combination of physical and biological processes using bentonite (R1) or kaolinite (R2), physical process only using bentonite (R3) or kaolinite (R4), and biological process only with NSW (R5) or enrichment medium (R6) (Table 1).

Observations of biofilm formation: Light Microscope, SEM, and TEM

At each sampling, subsamples of the clays in a liquid culture were analyzed for overall microbial activity (mainly biofilm formation) from the initiation to termination of the experiments. The biofilm formation was determined by light microscopy

scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Dense cultures were wet mounted on glass slides, and stained with 4',6-diamidino-2-phenylindole (DAPI), and observed with optical microscopy using a standard phase-contrast and epifluorescence microscope (Nikon NTF2A). Cultures were also fixed with 2% (vol/vol) glutaraldehyde in phosphate buffer at room temperature for 1 h, then post-fixed in 1% Osmium tetroxide at room temperature for 1 h, dehydrated in a graded ethanol series (50, 75, 95, 100%), critical point dried in CO_2 in a DCP1-CPD apparatus, mounted on carbon-coated copper stubs, and viewed on a JEOL JSM-5200LV scanning electron microscope. Samples for transmission electron microscopy were fixed and dehydrated as described above, except that they were not post-fixed in 1% Osmium tetroxide, mounted on copper specimen grids, and viewed using a JEOL JEM-2000EX transmission electron microscope.

Growth and enumeration of hydrocarbon degraders

Enumeration of the number of viable cells in liquid culture was determined by a serial dilution-agar plating procedure. This plate screening technique not only allowed the direct quantification of oil-utilizing microorganisms (particularly bacteria) in liquid cultures, but also enabled monitoring their microbial growth. Before sampling, the liquid cultures were slightly shaken for homogenization, to allow enumeration of the number of viable cells both suspended in the liquid medium and attached on clay minerals. The liquid cultures were serially diluted ten-fold in 0.85% NaCl solution. 0.1 ml of liquid was plated from each dilution onto nutrient agar (NA) plates consisting of 3.0 g of beef extract, 5.0 g of peptone, and 15 g of agar per liter of distilled water. NA is a general agar medium that supports the microbial growth especially bacteria and fungi, although we particularly focused on bacterial growth. All plates were incubated at 25 °C for 7 days. These methods did not distinguish between suspended and attached bacteria growth.

XRD analysis of mineral composition

The clay samples were analyzed by X-ray diffraction (XRD) analysis with a Rigaku Rinto 1200 X-ray diffractometer using CuKα and CrKα radiation, generated at 40 kV

and 30 mA, and using the 2θ/θ method and a scan speed 2°/min. XRD analysis was employed to determine whether there were relative changes in the basal spacings of bentonite and kaolinite during the removal of crude oil. Samples for XRD were prepared by spreading an aliquot of the suspended clays in ddH_2O over a 2.5-cm^2 area of a glass slide. The slides were air dried, and placed in a desiccator containing silica gel desiccant to prevent rehydration. To identify the clay minerals, the bentonite samples were expanded with ethylene glycol and then dried at room temperature before being analyzed by XRD.

FT-IR analysis of the adsorption capacity of clays and the removal of hydrocarbons

The depletion of crude oil (as hydrocarbon content) and the determination of the adsorption capacity of clays were evaluated by Fourier transform-infrared absorbance spectroscopy (Jasco FT-IR-610, MICRO-20) at wavenumbers 2700-3200 cm^{-1}. For the adsorption capacity of the clays, a portion of the clay samples in liquid culture was removed and dried at room temperature, and mixed with a small amount of KBr in a porcelain crucible, and then mechanically ground to a fine particle size. For hydrocarbon removal, approximately 5 ml of the liquid samples from each flask were placed into 20-ml vials, with Teflon-lined screw caps, containing carbon tetrachloride (CCl_4) to extract hydrocarbons. The samples for hydrocarbon analysis were dried by mixing with an equal volume of anhydrous sodium sulfate (Na_2SO_4) after treatment with CCl_4. To eliminate background disturbances due to polar non-hydrocarbon compounds, the samples were mixed with aliquots of florisil (magnesium silicate), before being analyzed by FT-IR. The removal efficiency of hydrocarbons was calculated with the following equation:

$$\text{Removal efficiency of hydrocarbon (\%)} = \frac{Co - Ct}{Co} \times 100$$

Where C_o is the initial concentration of hydrocarbon (mg/l) at time t = 0, and C_t is the

concentration of hydrocarbon (mg/l) in solution after adsorption and biodegradation at a sampling time = time t.

RESULTS

Crude oil removal (as hydrocarbon degradation)

Results in Figure 1 depict the hydrocarbon removal of various experimental systems with the time of incubation. It appears that after 7 days of incubation, there was a significant hydrocarbon removal in all the experimental systems, reaching the high level of approximately 86~98.5%, except for both R1 and R3, which had levels of only 49.6% and 19.8%, respectively. The highest removal efficiency of hydrocarbon was observed for R2 (98.5%), whereas the lowest was observed for R3 (19.8%). The extent of removal of hydrocarbons for each experimental system was in the sequence: R2 > R6 > R4 > R5 > R1 > R3, showing that microorganism had an effect on the removal of petroleum hydrocarbons. However, a combination of physical and biological processes using kaolinite (R2) was higher than that of R6, indicating that kaolinite supported this biodegradation and was not toxic and did not inhibit the degradation process. The biodegradation process increased in the presence of kaolinite, indicating that the removal of hydrocarbons was also as a result of the adsorption process by kaolinite. The same pattern was evident in R4. Bentonite had little effect on the removal of petroleum hydrocarbons, suggesting that bentonite inhibited the biodegradation process, as indicated by a low removal of hydrocarbons in R1 (49.6%) compared with that in R5 (86.3%). The adsorption potential of bentonite was also low or insignificant (R3 = 19.8%). Subsequently, the degradation processes continued for another 15 days, but, there was no significant degradation.

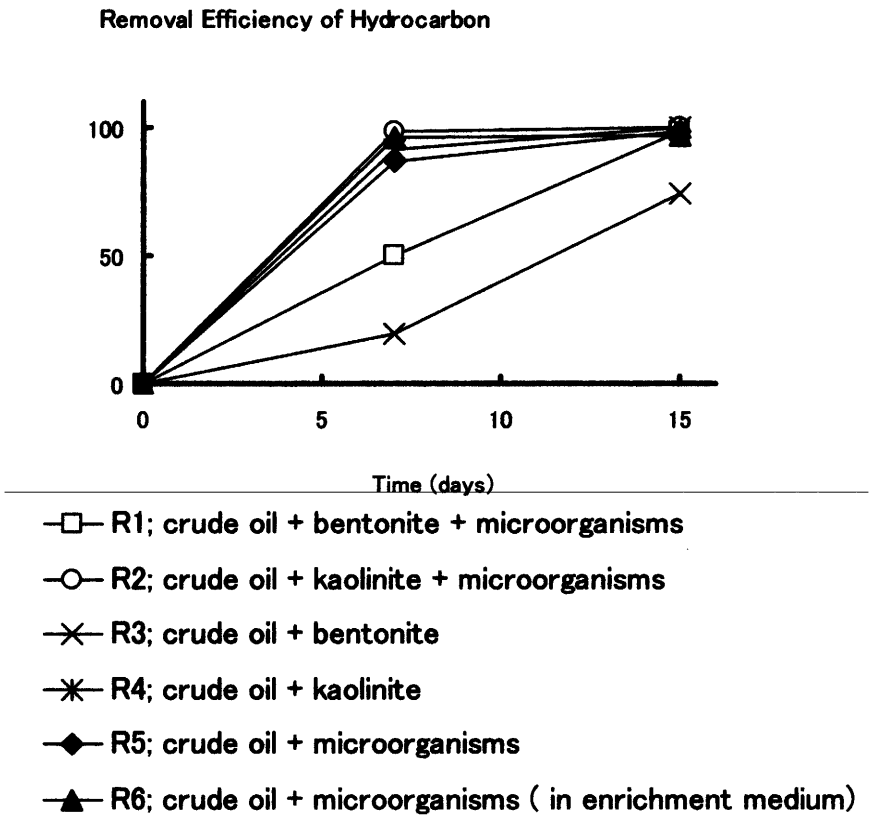

Figure 1 Removal efficiencies of crude oil (as hydrocarbon content) in all experimental systems with bentonite and kaolinite.

Microbial growth

Microbial growth was evaluated by a serial dilution-agar plating procedure and expressed as cfu/ml. The initial inocula added to flasks are given in Table 1. Bacterial growth predominated in all experimental systems throughout the incubation times, although fungi also grew (data not shown), but they were negligible in microbial enumeration. After 7 days of incubation, there was a tremendous increase in bacterial cell number of all experimental systems (Table 1). Microbial enumeration for R3 and R4 was not conducted, because they were abiotic controls that were already autoclaved three times to prevent microbial growth in crude oil, NSW, and clay minerals. Changes in the removal efficiency of hydrocarbons from 49.6% (on day 7) to 98.5% (on day 15) (see Figure 1) did not significantly affect the predominant bacterial populations in R1 (Table 1). In general, bacterial cell numbers (after 7 days and 15 days of incubations)

increased in both R1 and R2 (from 5.8 x 10^7 to 8.5 x 10^7 cfu/ml and from 4.6 x 10^6 to 5.8 x 10^7cfu/ml, respectively) but declining markedly (from 2.8 x 10^7 to 1.6 x 10^7 cfu/ml and from 1.6 x 10^9 cfu/ml to 1.4 x 10^8 cfu/ml, respectively) in both R5 and R6. These apparently reflected their greater dependence on the availability of support materials (i.e., bentonite and kaolinite) to perpetuate the biodegradation processes of hydrocarbons.

Table 1 Growth of hydrocarbon-degrading microorganisms during degradation of crude oil

	Experimental systems	0 day (cfu/ml)	7 days (cfu/ml)	15 days (cfu/ml)
R1	crude oil + bentonite+ microorganisms	**$3.6x10^2$**	**$5.8x10^7$**	**$8.5x10^7$**
R2	crude oil + kaolinite+ microorganisms	**$8.5x10^2$**	**$4.6x10^6$**	**$5.8x10^7$**
R3	crude oil + bentonite	–	–	–
R4	crude oil + kaolinite	–	–	–
R5	crude oil + microorganisms	**$9.6x10^2$**	**$2.8x10^7$**	**$1.6x10^7$**
R6	crude oil + microorganisms (in enrichment medium)	**$0.1x10^2$**	**$1.6x10^9$**	**$1.4x10^8$**

—; not measured.

Biofilm formation on clay surfaces

Differences were observed in the formation of biofilms in all experimental systems, demonstrating that an abundant biofilm formation occurred in both R1 and R2 (Figures 2, 3, and 4). However, the most abundant one was observed for R1 compared with that of R2, indicating that bentonite supported substantial extracellular activities of microbial cells in forming a biofilm. Generally, after 7 days of incubation, biofilm formation increased progressively in R1 and R2. These increased during the earlier week of incubation (15 days). Bentonite appeared to stimulate the production of polysaccharides by microbial cells more than kaolinite (Figure 2). SEM indicated that the cells were bound primarily on the edges of the clays, although some cells were

apparently also bound on the planar surfaces (Figure 3). Figure 3 exhibits scanning electron micrographs, at low magnifications, of biofilms formed on bentonite (A) and kaolinite (B) after 15 days of incubation. These micrographs show that the biofilms developed on the clays. The biofilms around the clays were densely packed and formed agglomerations. Biofilm formation on bentonite (R1) was generally larger and more serrated than on kaolinite (R2) (Figures 3a, 3b). Figure 3c and 3d show biofilm formation on bentonite and kaolinite at higher magnifications. The biofilms developed on the clays and a slight roughening of the surface of biofilms was observed. The biofilms on bentonite (R1) appear to be smoother than on kaolinite (R2). The size of the biofilm on kaolinite is smaller than on bentonite. Microbial growth apparently occurred mostly inside the biofilm matrix on bentonite, resulting in the disappearance of the clay in the SEM observation (Figure 3c).

TEM showed the same pattern of binding of microbial cells on the clays as did SEM; binding appeared to be particularly on the edges of the clays, although some cells also appeared to be bound on the planar surfaces (Figures 4c, 4d, 4e, and 4f). Subsequently, after 7 days of incubation, the cells grew and produced polysaccharides in relation to the biofilm formation. These resulted in the formation of the clay-cell complexes. It is apparent in Figure 4e and 4f that after 15 days of incubation, the cells are bound on the sites of the clays.

This apparent aggregation and the absence of microbial cells emanating from the sides of the clays suggested that the sites on the clays to which microbial cells were bound were clustered. However, the presence of few clustered sites would not have been detected, as the thickness of an individual microbial cell is insufficient to have been observed at the magnifications used. The results here were in accordance with those observed by light microscopy (see Figure 2), demonstrating that the polysaccharides exuded from microbial cells disrupted observation of each individual cell shape.

Mineral composition of clays

Varying the time of incubation from 0 day to 15 days did not substantially change the overall diffraction pattern profiles of both bentonite and kaolinite in any experimental systems (R1, R2, R3, and R4). Although the removal efficiencies of crude oil were high,

these did not result in an increase in the basal spacing of bentonite, and no expansion of kaolinite was observed, as kaolinite is a nonexpanding clay (Stotzky, 1986; Khanna et al., 1998) (Figures 5, 6), indicating that the crude oil did not intercalate either bentonite or kaolinite.

Adsorption capacity of clays

As described above, the adsorption of crude oil by both clays increased continuously with contact time under the conditions of agitation and neutral-alkaline pH (see Figure 1). For both adsorbents in the R1 and R2 experimental systems, the sorption capacity also increased with an increase in polysaccharides emanating from microbial cells (see Figure 2). The FT-IR analysis also provided evidence that microorganisms aided in the adsorption capacity of bentonite (Figure 5). However, microorganisms did not enhance the adsorption capacity of kaolinite (Figure 6), as shown by the low peaks at 2959 cm^{-1} (CH_3 group) and 2924 cm^{-1} (CH_2 group) (Figure 6). XRD data demonstrated that the total counts of basal spacing were reduced successively throughout the experiments for kaolinite in the presence and absence of microorganisms (Figure 6), but for bentonite only in the presence of microorganisms (Figure 5). In the absence of microorganisms, the total count of basal spacing of bentonite increased slightly after 7 days of incubation, but after 15 days of incubation, there was a sharp decrease in total count of basal spacing (Figure 5). The FT-IR data showed that the peaks of OH-structure were also reduced for bentonite in the presence and absence of microorganisms (Figure 5) but for kaolinite only in the presence of microorganisms (Figure 6).

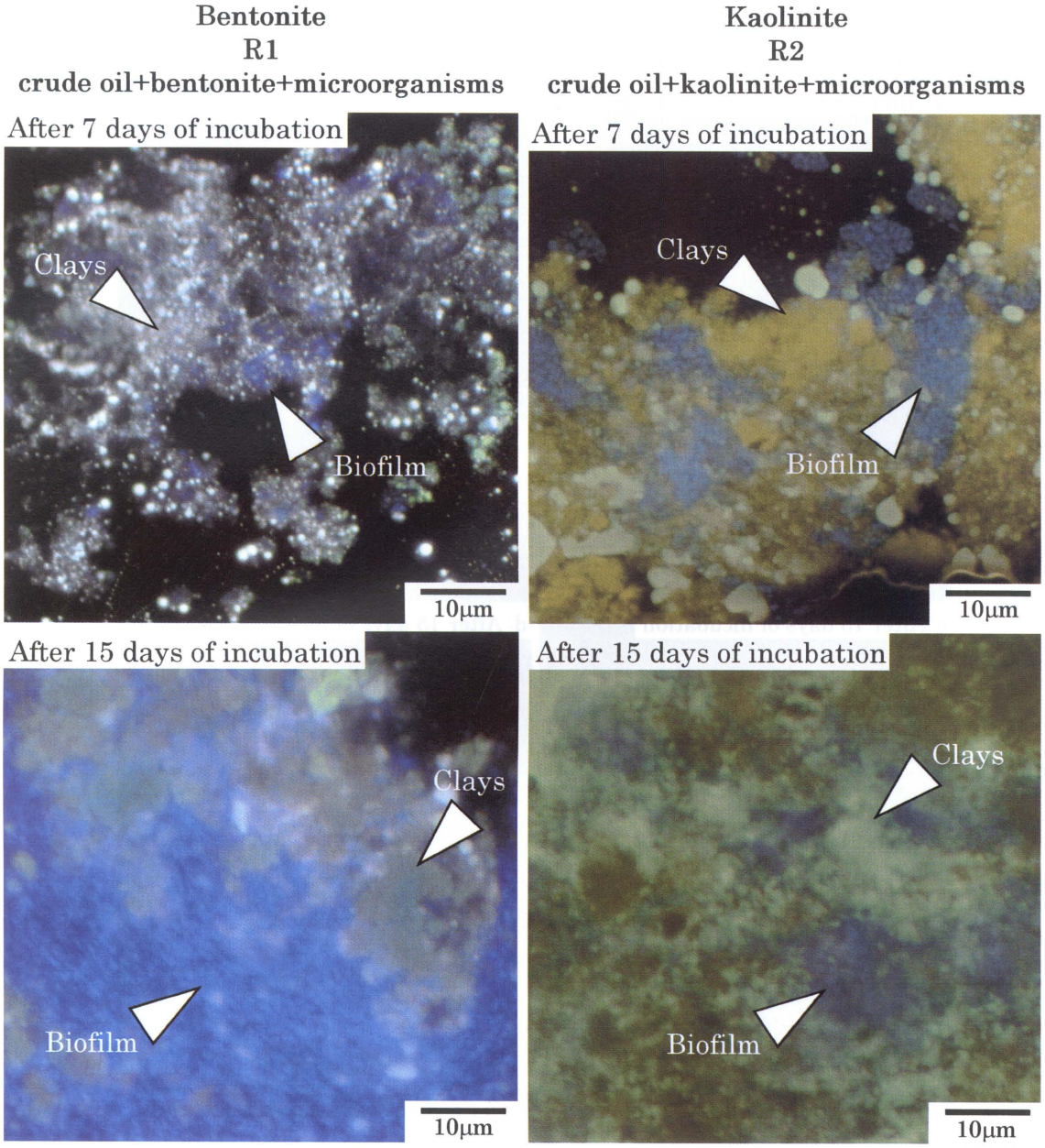

Figure 2 Optical microscopic images of microbial growth (biofilm formation) on bentonite and kaolinite during degradation of crude oil. R1; an experimental system using bentonite and microorganisms in degrading crude oil after 7 days and 15 days of incubation. R2; an experimental system using kaolinite and microorganisms in degrading crude oil after 7 days and 15 days of incubation. The arrows indicate the biofilm formation (designated by the blue color in figure) and clay minerals (designated by the brown color in figure if they adsorb a large quantity of crude oil and the green color in figure if they do not adsorb crude oil).

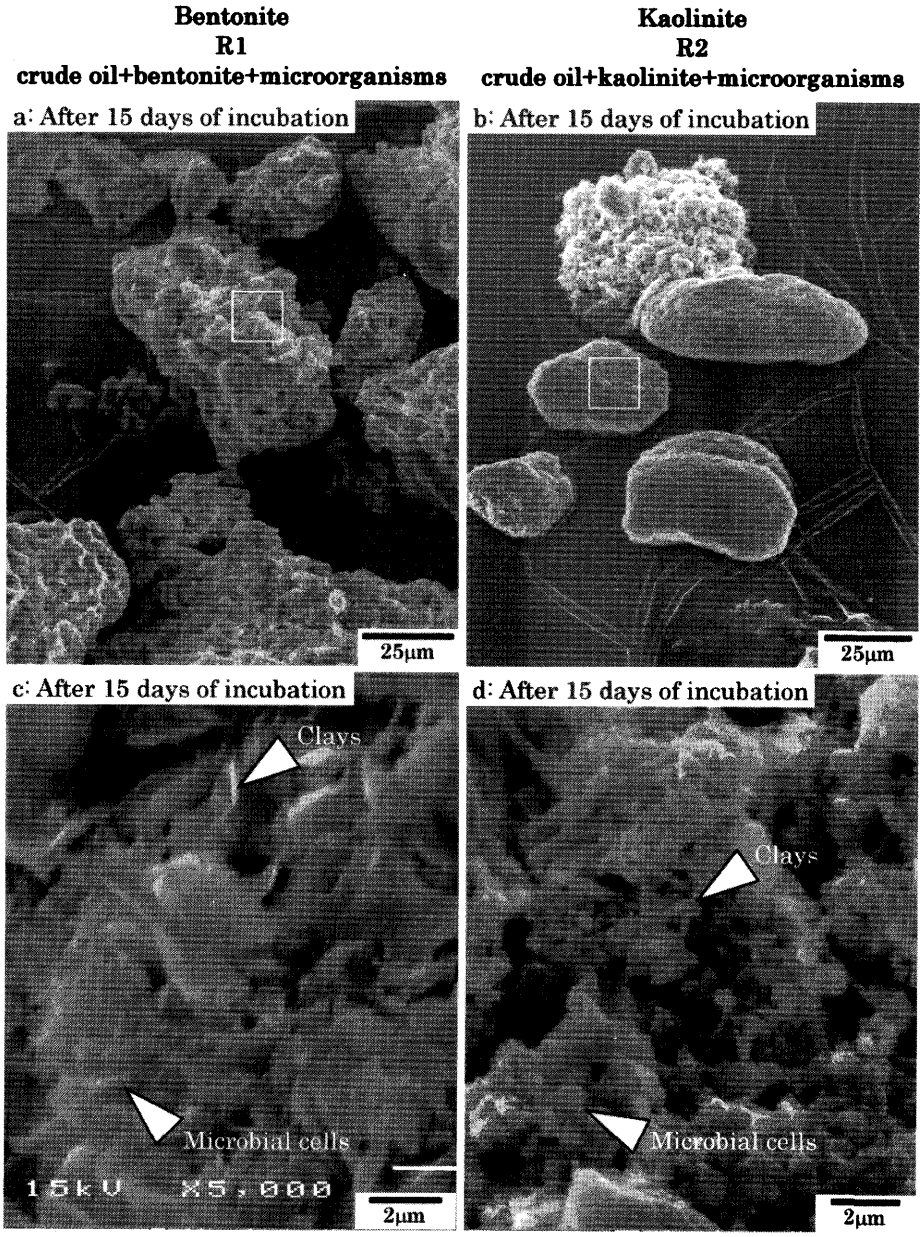

Figure 3 Scanning electron micrographs of microbial growth associated with biofilm formation on bentonite (a) and kaolinite (b) during degradation of crude oil. R1; an experimental system using bentonite and microorganisms in degrading crude oil after 15 days of incubation. R2; an experimental system using kaolinite and microorganisms in degrading crude oil after 15 days of incubation. The polysaccharides produced by microbial cells appear to be a cell aggregation along with the clays, where clay particles become embedded in the biofilm matrix. The magnified view of bentonite in the selected area of figure a (c), while the enlarged view of kaolinite in the selected area of figure b (d). The arrows indicate microbial cells and clay minerals

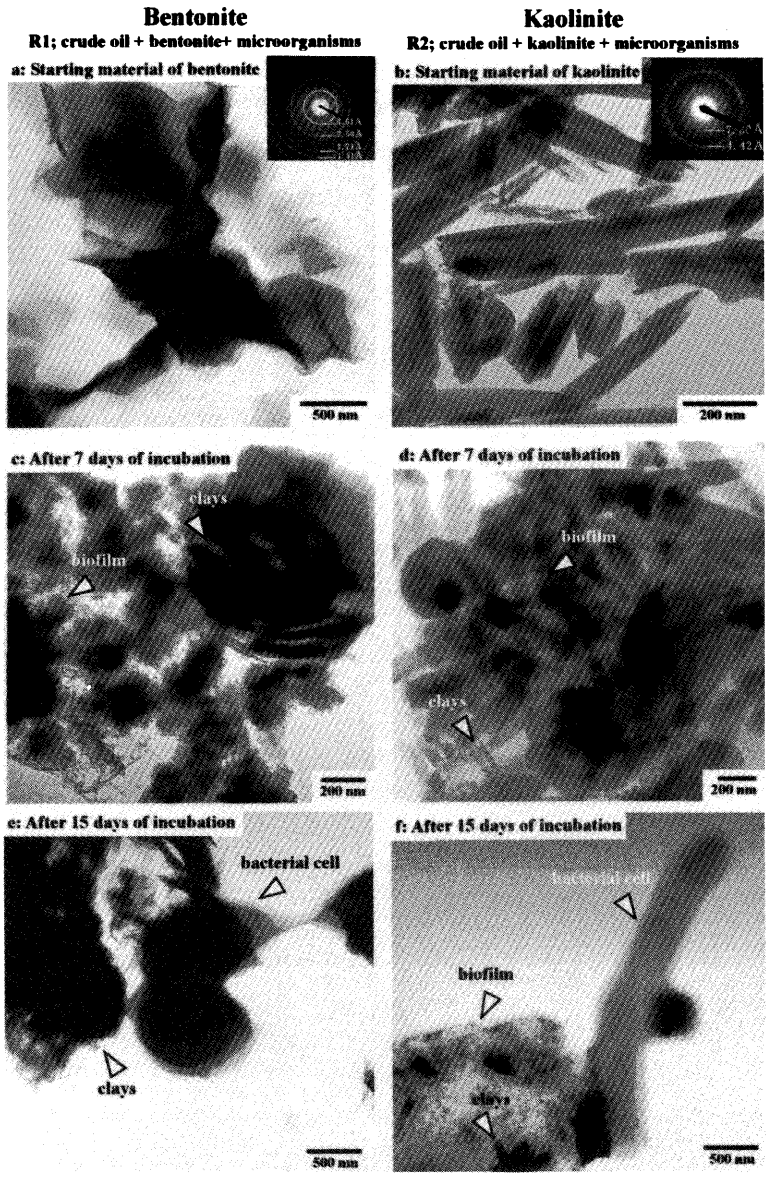

Figure 4 Transmission electron micrographs of bentonite and kaolinite initially and after microbial growth associated with biofilm formation during the degradation of crude oil. Starting materials of bentonite and kaolinite were also identified by electron diffraction patterns, respectively (inset; bentonite: 4.51 Å, 2.58 Å, 1.71 Å, and 1.51 Å, kaolinite: 7.30 Å and 4.42 Å). R1; an experimental system using bentonite and microorganisms in degrading crude oil after 7 days and 15 days of incubation. R2; an experimental system using kaolinite and microorganisms in degrading crude oil after 7 days and 15 days of incubation. The microbial cells appear to be bound on the edges and planar surfaces of bentonite and kaolinite. The arrows indicate microbial cells and clay minerals.

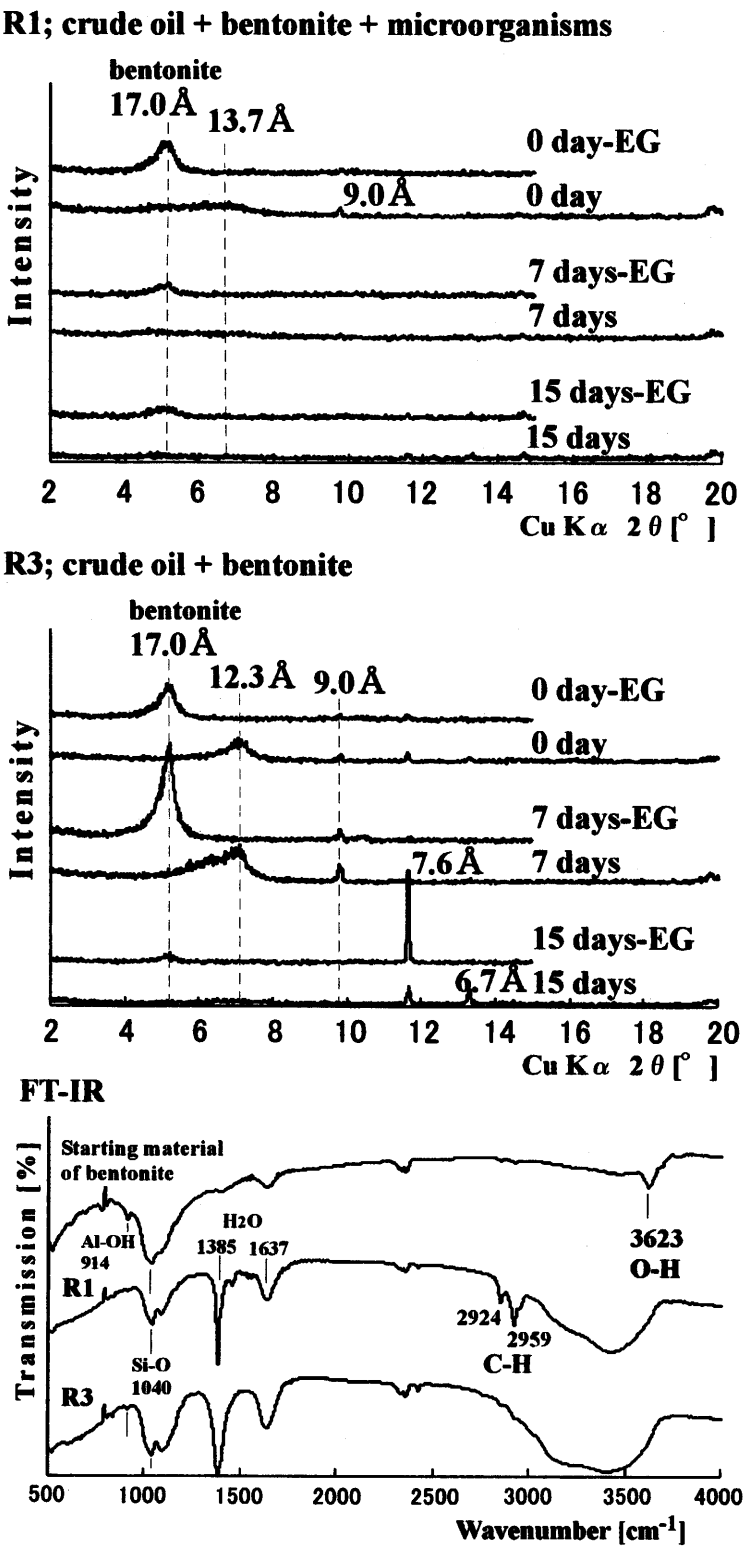

Figure 5 The resultant product profiles of crude oil degradation at experimental systems of R1 and R3 analyzed by XRD and FT-IR. R1; an experimental system using bentonite and microorganisms in degrading crude oil throughout the experiments (on 0 day, after 7 days and 15 days of incubation). R3; an experimental system using only bentonite in degrading crude oil throughout the experiments (on 0 day, after 7 days and 15 days of incubation). The analyses with ethylene glycol treatment (EG) were simultaneously conducted in the range of 2θ: 2°-15°. XRD data show a successive decrease in total count of basal spacing for R1 but not for R3, whereas the basal spacing of bentonite in both R1 and R3 is not altered. FT-IR spectra of starting material of bentonite, the experimental systems using bentonite and microorganisms (R1) and only bentonite (R3) in degrading crude oil after 15 days of incubation, indicating a change in the structure of bentonite after interacting with crude oil and microorganisms, and also showing its adsorptive capability extent of crude oil.

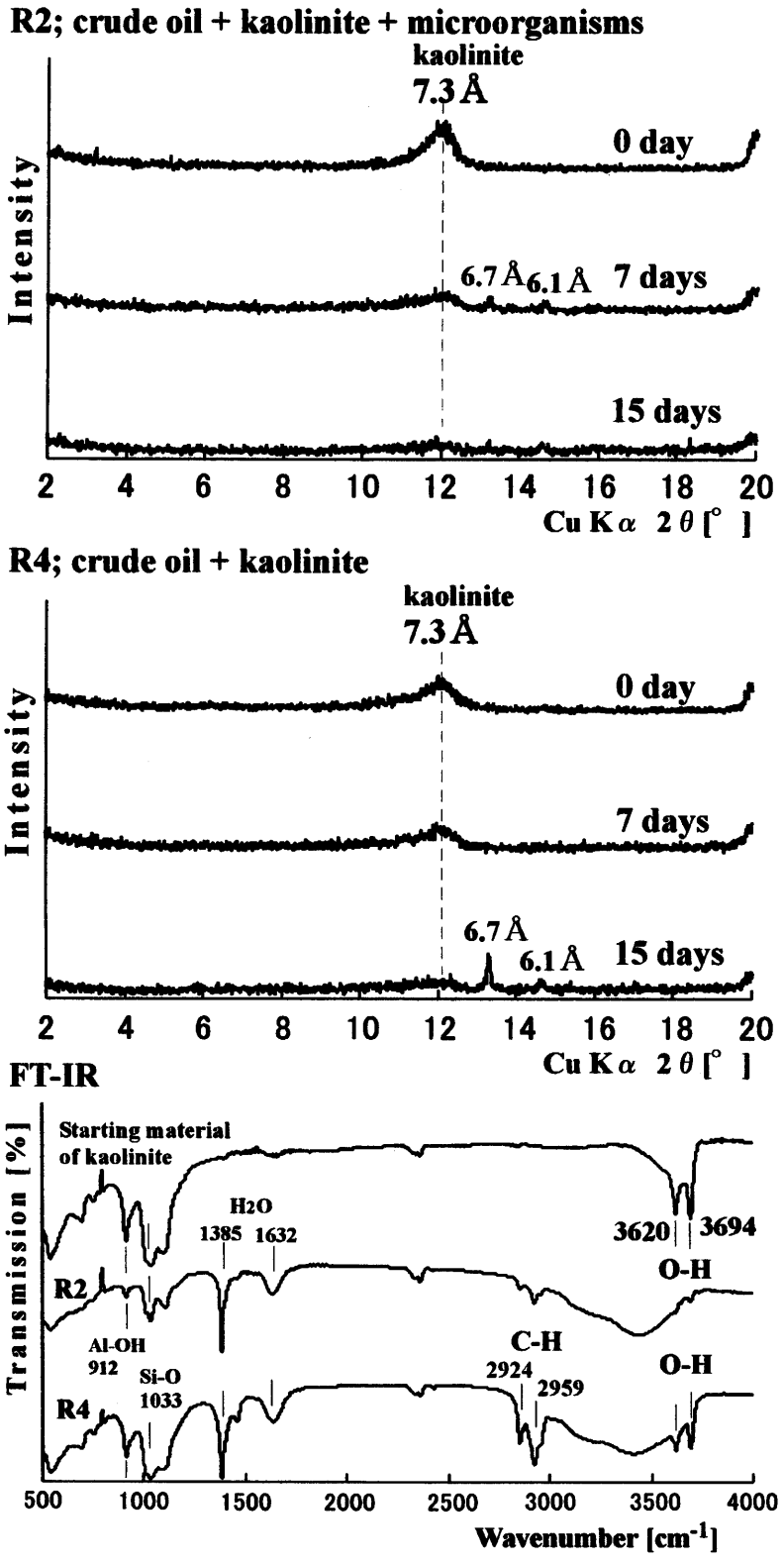

Figure 6 The resultant product profiles of crude oil degradation at experimental systems of R2 and R4 analyzed by XRD and FT-IR. R2; an experimental system using kaolinite and microorganisms in degrading crude oil throughout the experiments (on 0 day, after 7 days and 15 days of incubation). R4; an experimental system using only kaolinite in degrading crude oil throughout the experiments (on 0 day, after 7 days and 15 days of incubation). XRD data show a successive decrease in total count of basal spacing for both R2 and R4, whereas the basal spacing of kaolinite in both R2 and R4 is not altered. FT-IR spectra of starting material of kaolinite, the experimental systems using kaolinite and microorganisms (R2) and only kaolinite (R4) in degrading crude oil after 15 days of incubation, indicating a change in the structure of kaolinite after interacting with crude oil and microorganisms, and also showing its adsorptive capability extent of crude oil.

DISCUSSION

Clay minerals have been widely employed as adsorbents for the removal of many kinds of pollutants (Chaerun et al., 2002a; Chaerun et al., 2002b). However, there are few studies about their application for the removal of oil spills.

Bentonite and kaolinite as adsorbents

Both bentonite and kaolinite were highly effective adsorbents for the removal of crude oil spilled from the *Nakhodka* oil tanker (Figure 1). Kaolinite was a more effective adsorbent than bentonite. However, as the contact time increased, (R1) the removal efficiency of bentonite of crude oil increased to somewhat as high as kaolinite (R2) (after 15 days of incubation). However, the removal efficiency of bentonite alone of crude oil (R3) did not attain the same extent as R1, R2, and R4. It was presumed that there was a synergism between the clays and microorganisms in removing the crude oil. Additionally, SEM and TEM observations showed that the particle size of bentonite was larger than kaolinite. Also, the bentonite employed in this study was a conventional one (i.e., unamended bentonite). These reasons might contribute to the low adsorptive extent of bentonite compared with kaolinite. It was also possible that bentonite had a smaller pore-size than kaolinite, resulting in a low adsorptive capacity for the removal of crude oil which was of relatively large molecular size (Cooney, 1999). It was also stated by Cooney (1999) that the external area defined the amount of contact area between the clays and a surrounding solution, and the rate of transfer of adsorbable molecules from the fluid phase to the clay phase would be proportional to the external area; in contrast, the internal surface area determined the amount of adsorption that could occur at equilibrium. The external area of kaolinite might be greater than that of bentonite, rendering the kaolinite had more rapid rate of transfer of crude oil than bentonite. FT-TR data also reinforced these assumptions (see Figures 5 and 6). When adsorbing a large molecule (e.g., crude oil), much of the internal surface area might not be accessible. Additionally, Cooney (1999) also reported that the molecular structure of the adsorbate was also an important factor. For example, (1) aromatic compounds are usually more adsorbable than aliphatic compounds of a similar molecular size, (2)

branched-chain molecules were generally more adsorbable than straight-chain molecules, and (3) the effect of a substituent group depended much on the position (e.g., ortho, meta, para, other) where it was introduced. The chemical composition of crude oil used in this study (from the *Nakhodka* oil spill) was mainly aliphatic hydrocarbon (n-alkanes of C_9-C_{30}, in which n-eicosane (n-$C_{20}H_{42}$) was the most abundant compound), whereas the content of aromatic compounds was low (Tazaki et al. 1997a; Tazaki et al. 1997b; Shibata et al. 1997; Itagaki and Ishida 1999). It was also reported that the amount of adsorption of phenols on clay-organic complexes was dependent on the relative energies of adsorbent-adsorbate and adsorbate-solvent interactions (Mortland et al., 1986).

Bentonite and kaolinite as microbial growth-support media associated with biofilm formation

Bentonite was a more suitable support material/media for microbial growth in relation to biofilm formation than kaolinite. Microbial cells bound on the clay minerals retained the ability to proliferate (Table 1) and biodegrade crude oil (Figure 1). The immobilization of microbial cells on the clay minerals might cause an increase in microbial activity and stability due to more favorable microenvironmental conditions (Shuler and Kargi, 1992). The initial step of degradation of crude oil requires that microorganisms dissolved the crude oil. Subsequently, dissolution and diffusion of dissolved hydrocarbons to cells occurred, followed by uptake, via active or passive transmembrane transport, and invagination of hydrocarbon non-aqueous-phase liquid (NAPL) into cells (Finnerty and Singer, 1985; Holden et al., 2002) with subsequent intracellular metabolism of the hydrocarbon inclusions. Finally, the microbial cells produced surface-active compounds, such as surfactants (biosurfactants) and emulsifiers (bioemulsifiers), that increased the local pseudosolubility of hydrocarbons and, thus, improved mass transfer to biodegrading microorganisms (Hommel, 1990; Miller, 1995; Holden et al., 2002). By this process, it was also possible that the resultant product of the biodegradation of crude oil resulted in the cleavage of long hydrocarbon chains to shorter and simpler ones. This probably had an effect on adsorption of crude oil by bentonite, as shown by increase in the adsorption power for crude oil (R1) after 15 days of incubation (Figure 1), as the small pores of bentonite, which did not initially admit

the large molecule of crude oil, admitted them due to the resultant smaller molecules The thick biofilm formation on bentonite and the biosurfactants produced by microbial cells probably increased the adsorptive capacity of bentonite. This phenomenon reflected on the amended clays. Lee et al. (1990) reported that, in general, the sorptive capacity of amended clays was greatly increased when compared with that of unamended clays. It has been reported that bentonite increased the growth rate of rhizobia introduced into sterile soil, and that carbon was used more efficiently during growth in bentonite -amended than in unamended loamy sand (Heijnen et al., 1993).

Effect of crude oil and biofilm formation on the composition and adsorption capacity of bentonite and kaolinite

The results of XRD, SEM, and TEM indicated that crude oil and microbial cells did not intercalate the clays but were bound primarily on the edges and outer planar surfaces of the clays. It was postulated that the crude oil and microbial cells formed one type of complex with the clays: they were adsorbed on the external surfaces. One possible reason for this phenomenon was that microbial cell utilized in this study had a large size, which did not enable an intercalation of the clays. Our results are in agreement with the earlier observations of Khanna et al. (1998) that the DNA (from *Bacillus subtilis*) adsorbed at equilibrium on montmorillonite and kaolinite was tightly bound and was protected. By contrast, another work reported that DNA (8.9×10^6 daltons in size) intercalated the montmorillinite by forming two types of complexes with montmorillinite: in one, the nucleic acids were adsorbed on the external surfaces, and in the other, the nucleic acids were adsorbed on both external and internal surfaces (Greaves and Wilson, 1970). Obviously, the microbial availability essentially aided and supported the adsorption process of crude oil by the clays (primarily bentonite), which as a consequence, resulted in an increase of crude oil removal. The mutuality between microorganisms and the clays obviously would be of benefit to the removal of crude oil on the application to bioremediation process. In addition, bonds between oil and bentonite resulted in a lost of expandability of bentonite, which ultimately altered the characteristic of bentonite to become similar to kaolinite (as nonexpanding clays), eventually resulting in the decreases of total count of basal spacing after interacting with

crude oil. Our results confirmed the findings obtained by Banat et al. (2000), that the presence of cyclohexane (non-polar compound) destroyed the crystalline structure of bentonite and, consequently, converted it to an amorphous form, which eventually resulted in a substantial increase in the surface area of bentonite.

According to the FT-IR data, the peaks of OH-structure were also reduced for bentonite in the presence and absence of microorganisms (Figure 5) but for kaolinite only in the presence of microorganisms (Figure 6), indicating that both microorganisms and crude oil were responsible for the chemical bonding of the OH-structure in bentonite, whereas in kaolinite only microorganisms were responsible. Furthermore, FT-IR data also indicated that microorganisms which excreted a large amount of polysaccharides in association with biofilm formation, had an essential role in the adsorption of crude oil on bentonite (Figure 5) but not on kaolinite (Figure 6). Optical microscope observations corroborated these results (Figure 2).

CONCLUSIONS

Degradation of crude oil apparently resulted from a favorable synergism between clay minerals and hydrocarbon-degrading microorganisms. The ubiquitous presence of microorganisms in crude oil and introduced clays indicated that clays were not a limiting factor for the growth of microorganisms in degrading crude oil. Instead, the presence of the clays resulted in a growth of microorganisms that produced polysaccharides bringing about biofilm formation (especially with bentonite) which accelerated the degradation of crude oil. The clay minerals had a role as adsorbents and microbial growth-support media in enhancing the degradation of crude oil.

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