Development of Microorganism Enrichment Culture Method

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1. R&D Objectives

The petroleum industry in Japan is confronted with the issue of rationalization of facilities such as oil refineries, oil terminals and oil service stations. Consequently, in making effective use of land remaining after such facilities have been removed, there is concern over the presence of oil-contaminated soil.

Various types of soil remediation technologies are currently being developed, but those aimed at petroleum consist mostly of technologies for light hydrocarbons. With respect to oil-contaminated soil, including heavy oil, very few present-day technologies boast of remediation results. What is more, advances in the development of remediation technology for heavy-oil-contaminated soil center on physical and chemical purification technologies. Accordingly, in order to develop biological remediation technology using microorganisms, which is expected to further reduce costs, work is being directed toward the development of technologies centering on microorganism enrichment culture method and toward reduction of remediation time periods. Moreover, by combining such technologies with extant physical and chemical remediation methods, optimum technology for soil remediation will be established in consideration of remediation time period for oil-contaminated soil, remediation levels reached, economy, and so on.

2. R&D Contents

2.1 Accomplishments in FY 2001

2.1.1 Development of Microorganism Enrichment Culture Method

Based on results obtained in FY 2000, conditions were investigated respecting the following issues, which were clarified for establishment of an efficient collection technology for valuable microorganism strains.
There are locations where liquid passageways appear in the column soil layer, cultivation liquids undergo drifting, and supplies of nutrients and oxygen are inadequate. → Investigation through soil churning

Special nutrient resources are inadequate in cultivation liquids. → Periodic replacement of culture medium

Obstructive intermediates are produced, causing reduction of activity by soil microorganism strains. → Periodic replacement of culture medium

There are no strains in the soil used that decompose Polycyclic aromatics. → Addition of microorganism preparations sold on the market

2.1.2 Small-scale Experiments Using Microorganism Collection Technology

As a small-scale remediation experiment, microorganism enrichment culture method was applied to the land farm method, compared with conventional methods and investigated for superiority.

2.1.3 Investigations to Determine Detailed Status of Contaminated Soil

Based on results in FY2000, a radar detection system was used to determine the status of contaminated soil, and a study was conducted to determine features in using the system for surveying contamination states in detail. Oil contamination concentrations were analyzed in three dimensions, volumes of contaminated soil were determined, and comparisons were made with the boring method in terms of economy.

3. R&D Results

3.1 Analysis Methods

3.1.1 TPH Measurement Method

At the Kamiizumi Branch Office, studies are underway for establishing accurate quantification methods for light to heavy oil in the soil. In the previous fiscal year, the JIS method was revised into one of high quantification and few disparities; it has been investigated and used provisionally as a quantification method for Total Petroleum Hydrocarbons (TPH) until a method such as the aforementioned can be established. In comparison to the conventional JIS method, this method offers improved precision in analysis, but it is poor in workability because increased operations are needed for such things as filter paper cleaning. The number of samples that can be analyzed by one person per day stands at about 8.

TPH analysis at good efficiency must be advanced since in the current fiscal year, there has been an increase in the number of experiments, such as those for evaluating oil decomposition involving column collection and small-scale remediation experiments (land farm method).

Accordingly, studies were done on application of a TPH simplified analysis method used by K.K SVC Tokyo, and it was confirmed that the method can be used in the undertaking. The method is described below.
(1) A prescribed volume of pre-dried soil is placed in a centrifugal precipitator, a prescribed volume of carbon tetrachloride is added, shaken and extracted for 10 minutes, then subjected to centrifugal separation for 10 minutes.

(2) Surface-layer carbon tetrachloride extraction liquid is put into a 10 mm cell for IR, and TPH is measured.

(3) Concentration is determined from an analytical curve established in advance.

Artificially contaminated soil was created at each concentration level, using each type of medium and heavy oil, and such things as disparities, repeatability and recovery rates were checked. As a result, it was confirmed that disparities were kept to within 2%. Recovery rate was 90% of the additive theoretical value.

3.1.2 Microbe Count Measurement Method

In the present R&D, the purpose of taking a microbe count is to collect normal microorganisms strains that have the capacity to decompose oil in contaminated soil and to accelerate the contaminated oil decomposition process. Microbe concentration in the soil, therefore, serves as an index of contaminated oil decomposition speed.

In the cultivation of regular microorganisms, there are many cases, when taking microbe counts in liquids, in which measurements can be made from turbidity, yielding results in a short time through simple operations. In the present R&D, however, it was difficult to measure microbe concentration by this turbidity method because in both the microorganism enrichment culture method and the liquid culture method, there are soil particles in the liquid. In the present R&D, therefore, attention was focused on adenosine triphosphate (ATP), a high-energy substance that is necessary for life activities and that exists in living microorganisms. Microbe count was determined by measuring this ATP.

The measurement principle can be described as follows. Luciferin, a light-emitting substance, and ATP bond on a single molecule basis, and faint light is produced by a 2-stage reaction. What catalyzes this reaction is luciferase enzyme. The amount of emitted light is dependent upon the volumes of ATP, luciferin and luciferase, but if there is an excess of luciferin and luciferase, the amount of light is dependent upon ATP. There is a direct linear correlation between ATP and the amount of light emitted.

In this measurement method, a fixed volume of sterile water is added to a fixed amount of soil, and microorganisms in the liquid are then separated by a shaking method. In liquid diluted from 1 to about $10^4$, each colony count (microbe count) is measured and the amount of emitted light is determined in accordance with the aforesaid principle. Microbe concentration is determined from a pre-drawn analytical curve of emitted light versus microbe count, based on diluted liquid with a magnitude of emitted light that falls within the scope of the analytical curve.

3.1.3 GC Analysis Method

In the previous fiscal year, the GC method for analysis of heavy oil was investigated. Conditions were established for obtaining a gas chromatogram for the purpose of observing remediation speed and the reduction in each type of hydrocarbon. In the present fiscal year, in order to obtain a rough quantitative grasp, using GC, of conditions for reducing each type of hydrocarbon, the conditions for separation of saturated hydrocarbons and polycyclic aromatic hydrocarbons were determined, and separated entities were taken as samples for GC and GC-MS.
GC analysis conditions are shown in Table 3-1.

**Table 3-1: GC Analysis Conditions**

<p>| | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Unit</td>
<td>SHIMADZU GC2010</td>
</tr>
<tr>
<td>Injector</td>
<td>Injection volume: 1 µ l</td>
</tr>
<tr>
<td>Inlet</td>
<td>Split: 2:1</td>
</tr>
<tr>
<td></td>
<td>He</td>
</tr>
<tr>
<td></td>
<td>Heater: 320°C</td>
</tr>
<tr>
<td></td>
<td>Total flow: 8.2 ml/min</td>
</tr>
<tr>
<td>Column</td>
<td>Linear speed mode</td>
</tr>
<tr>
<td></td>
<td>Linear speed: 38 cm/sec</td>
</tr>
<tr>
<td></td>
<td>DB-5MS (Length 30 m × Inner diameter 0.25 mm × Film pressure 0.25 µm)</td>
</tr>
<tr>
<td>Oven</td>
<td>50°C (3 min) - 7°C/min - 320°C (11 min)</td>
</tr>
<tr>
<td>Detector</td>
<td>350°C</td>
</tr>
</tbody>
</table>

3.1.4 GC MS analysis method

Like the aforesaid GC method, the GC-MS method was investigated so as to yield favorable separation conditions. GC-MS operational conditions are presented in Table 3-2.

Shown in Figure 3-1 and Figure 3-2 are the chromatograms of standard products among saturated hydrocarbons and polycyclic aromatic hydrocarbons, respectively, assuming the conditions referred to above.

**Table 3-2: GC-MS Operational Conditions**

<p>| | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Unit</td>
<td>Agilent 5973N/6890Plus</td>
</tr>
<tr>
<td>Injector</td>
<td>Injection volume: 3 µ l</td>
</tr>
<tr>
<td>Inlet</td>
<td>Split: 10:1</td>
</tr>
<tr>
<td></td>
<td>He</td>
</tr>
<tr>
<td></td>
<td>Heater: 250°C</td>
</tr>
<tr>
<td></td>
<td>Total flow: 23.8 ml/min</td>
</tr>
<tr>
<td>Column</td>
<td>Constant mode</td>
</tr>
<tr>
<td></td>
<td>Linear speed: 38 cm/sec</td>
</tr>
<tr>
<td></td>
<td>HP-5MS (Length 30 m × Inner diameter 0.25 mm × Film pressure 0.25 µm)</td>
</tr>
<tr>
<td>Oven</td>
<td>50°C (3 min) - 7°C/min - 320°C (5 min)</td>
</tr>
<tr>
<td>MSD</td>
<td>280°C</td>
</tr>
</tbody>
</table>
3.2 Microorganism Enrichment

3.2.1 Outline of Microorganism Enrichment Instrument and Culture Conditions

(1) Microorganism enrichment instrument

In consideration of ease in soil sampling, assembly, cleaning procedures, etc., the microorganism enrichment system to be used was designed so that the column component and the liquid storage tank below do not form a single body but can be separated.
In view of the frequency of sampling and the magnitudes of samples taken, the column to be used must have enough capacity to insure that there is no impact from sampling. On the other hand, because the oil concentration drops over time due to decomposition by microorganisms, in the analysis of oil over time, there must be enough oil to insure the limit concentration for analysis. Given these considerations, the column capacity used was 300 ml and the capacity of the liquid storage tank below was 1000 ml. In addition, a stainless steel support screen of 120 mesh, measuring 25 mm in diameter, was installed under the column so that soil will not fall into the liquid storage tank.

Based on the results of a preliminary investigation, it was decided that the ratio of column diameter to column height would be 70 mm (inner diameter) × 120 mm (height). The column layers were filled with 300g (approx. 270 ml in cubic volume) of dried soil. Cubic capacity of the liquid storage tank was 1000 ml.

Inlets were added to the bottom storage tank for pH electrode and for dissolved oxygen (DO) electrode. On the flange over the liquid storage tank is an outlet for air bleeding tube.

The liquid culture storage tank is mounted on a magnet stirrer, and the liquid culture in the tank is churned by the stirrer. An outline drawing of the microorganism enrichment instrument is given in Figure 3-3.

![Figure 3-3: Outline Drawing of Microorganism Enrichment Instrument](image)
(2) Microorganism culture conditions

1) As nutrient substances other than carbon sources required for culture soil composition, for microorganism propagation and for life activities, a nitrogen source of ammonium nitrate (C vs N weight ratio = 100:10) was added to a mixture of disodium hydrogen phosphate (dodecahydrate) and dipotassium hydrogen phosphate, serving as phosphorous source (C vs P weight ratio = 100:1). Iron, magnesium, zinc sulfate, calcium chloride and manganese chloride were added as trace amounts of heavy metal. The other trace amount of organic constituent used was yeast extract.

2) Operating conditions

- Liquid circulation speed: 5 to 10 ml/min
- Cultivation temperature: Prescribed temperature (Normally 30°C)

3.2.3 Investigation of Microorganism Enrichment Factors

(1) Impact of churning

In the investigation of each condition, it is essential to confirm whether there are differences in results due to the condition. Accordingly, 3 columns were used in an investigation of contaminated soil in order to identify disparities during column cultivation.

Cultivation took place over 6 weeks, and TPH, microbe count and pH were determined once per week. Maximum disparity in TPH among the 3 columns was not more than 9.4%. Microbe counts were also roughly on the same order of magnitude. Given that disparities in regular microorganism experiments are at 10%, it was judged that these devices could be used equally well for investigating conditions.

Because it was feared that there might be culture liquid drifting, comparison was made between cases in which churning was performed only once per week during soil sampling and cases in which the soil was churned once every 2 or 3 days in order to prevent drifting. Over a collection period of 6 weeks, however, virtually no differences were noted in any of the weeks in TPH or microbe count. Consequently, it was judged that with the present device, there will be no culture liquid drifting if churning is implemented about once per week. Hence it is conjectured that the liquid travels through the column soil.

(2) Impact of culture medium replacement

In cultivation experiments conducted last year, it was learned that remediation stops in the fifth week of enrichment. Among the causes proposed is that specific nutrient sources are fully consumed and become inadequate during the period of column enrichment. Another suspected cause is that intermediate metabolites are produced, obstructing the microorganism strains so that they stop propagating. In order to investigate these causes, the culture liquid was replaced with fresh medium once per week over a 6-week period during which enrichment took place.
It was found that between cases in which the culture medium was replaced and cases in which it was not replaced, there were virtually no differences in TPH value per week and roughly similar patterns of decline were manifested. In experiments in which the culture medium was not replaced, trends in the concentrations of ammonia, nitric acid and phosphorous in circulating culture liquid were measured, and it was found that these substances remained in adequate amounts even after 6 weeks. To confirm the presence of organic acids in the circulating culture liquid during experiments with culture medium not replaced, the culture liquid was measured by infrared spectrophotometer on the sixth week. It was discovered that when petroleum was used as the raw fuel, there were no substances containing carbonyl or ketone, for instance, which are regularly produced as intermediates. Measurements were also taken of residual oil using infrared spectrophotometer, but there were no substances containing carbonyl or ketones. These findings indicate that no obstructions to cultivation are caused by intermediates.

3) Impact of microorganisms sold on the market

Since it was suspected that the microorganisms in the soil used might not be able to decompose polycyclic aromatics, a microorganism preparation sold on the market that decomposes heavy oil was added, and its impact was investigated. It was found that there were no differences in the pattern of decline in TPH even after 12 weeks of cultivation. This suggests that in the soil used there were microorganisms equivalent in capacity to the microorganisms on the market that decompose heavy oil.

4) Impact of pH

An investigation was made of the impact of pH, a key factor in the propagation of microorganisms in soil, on oil decomposition activity and on propagation speed. In experiments using soil artificially contaminated with C heavy oil and n-hexadecane, when the pH was not controlled, if the pH was initially at 7, it climbed to about 8 several weeks after the start of collection, dropped to 5 or 6 on the acidic side, then rose again to the alkali side.

When pH was controlled at 7.2, the oil decomposition speed was faster with both C heavy oil and n-hexadecane than in the case of no control. Microbe count was about the same both when pH was controlled and when it was not controlled. Hence it can be concluded that pH has more of an impact on oil decomposition by microorganisms than on microorganism propagation.

3.3 Verification Tests on Small Scale

3.3.1 Test method

As a means of conducting small-scale tests on remediation, experiments in decomposition were performed by the land farm method (temperature held at 30°C, mixing and churning once every 2 ~ 3 days, and moisture content held at 15%) on approximately 10,000 mg/kg of contaminated soil and on pseudo contaminated soil with hydrocarbons added in container (230L × 135W × 120 mm H). Prescribed volumes of nutrient salts were added, including Mg, Fe, Mn, Zn, Ca and yeast extract, along with carbon, nitrogen and phosphorous at the ratio (C:N:P = 100:10:1) in accordance with the amount of hydrocarbons in the soil.
3.3.2 Test Results

(1) Impact of added quantity of enriched microorganisms

Microorganisms that had been cultured in column enriched soil over a two-week period of column enrichment were added to dry soil in a quantity of 20-30 wt%. Experiments without enriched soil were prepared as control.

The remediation process is depicted in Figure 3-4. The figure shows that the remediation speed has clearly accelerated in the case where enriched soil was added to contaminated soil at 20 or 30 wt%. In the case of 30 wt% addition, the TPH concentration in the 4\textsuperscript{th} week dropped from 9,800 ppm to 6,000 ppm. In the control experiments with no enriched soil added, it took 20 weeks for TPH concentration to drop to 7,000 ppm. In contrast to this, the decomposition speed in the aforesaid case accelerated 5 times or more.

The purpose of the present R&D was to shorten the remediation period to one-half, but by adding enriched soil with a 2-week enrichment period, the remediation period could be reduced to 6 weeks as opposed to 20 weeks without enriched soil and thus the remediation period was actually reduced to one-third or less than its previous duration.

In the future, given that the magnitudes of added soil are not realistic, it will be necessary to identify the microorganism colony so as to determine the optimum addition time period and microbe magnitude; it will also be necessary to determine the minimum amount of addition from the microbe magnitude. These topics are currently being investigated. In addition, control factors will have to be studied so that the repeatability of the experiments is assured.

![Figure 3-4: Impact of Added Quantity of Collected Soil](image-url)
(2) Impact of enrichment time period for added microorganism strains

In enriching microorganism strains that decompose heavy oil, it is suspected that the dominant microorganism colonies differ in terms of enrichment time period. Next, therefore, enriched soil in which the enrichment time period had been changed was used to investigate impacts on heavy oil decomposition speed in the land farm. Experiments were conducted in the same manner as described above. The results are shown in Figure 3-5.

![Figure 3-5: Impact of Enrichment Time Period for Added Microorganism Strains](image)

In Figure 3-5, no differences could be noted in the rate of decomposition of the oil component in contaminated soil to which enriched soil had been added after the column enrichment time periods had been changed to 1, 2 and 3 weeks.

3.4 Comparison of Solid Phase Cultivation Method With Liquid Cultivation Method

To clarify the characteristics of column enrichment, microorganisms that decompose heavy oil were enriched. The heavy oil decomposition process was determined by means of the liquid culture method, the traditional means of enriching microorganisms, and comparisons were made with the column enrichment method.

3.4.1 Experiment Method

Each type of oil (C heavy oil, light oil) was added to a 500 ml shouldered flask at 1% concentration, together with 0.1g of soil and 70 ml of liquid medium, and cultivation tests were performed in a reciprocating shaker of constant temperature (120 times/min., 30°C).
3.4.2 Results of Decomposition via Liquid Culture

Results are given in Figure 3-6. About 60% decomposition of C heavy oil was noted in the fourth month and 70% in the fifth month. Microbe count was roughly the same in the fourth and fifth month as in the third month. In control -1 in the fifth month (pure water), oil decomposition was about 3% and with no nutrient sources, it was found that there was virtually no propagation. In control -2 (sterilization), decomposition was about 9%. This difference is ascribed to the fact that because sterilization (120°C x 20 min.) took place by autoclave as pretreatment before cultivation in control -2, the distillate evaporated at 120°C. In any event, it was discovered that when C heavy oil at 30°C is churned together with oxygen in a liquid medium, there is not much change.

3.4.3 Comparison of Heavy Oil Cracking Process with Solid Phase Cultivation Method and Liquid Culture Method

To facilitate enrichment of microorganisms in soil at good efficiency, an environment homologous to that of soil was created in columns. The present R&D was targeted at enriching microorganisms of high potential for decomposing heavy oil. Accordingly, a comparison was made to determine how hydrocarbons are decomposed in the liquid culture method, the common method of enriching microorganisms, as opposed to the solid-phase cultivation method in soil.
Remnant oil samples for TPH analysis from both the land farm method (solid phase cultivation) and the liquid culture method were divided into saturated hydrocarbons and polycyclic aromatic hydrocarbons, and for each separated sample, temporal changes were investigated using the GC MS method.

In this retention time, saturated hydrocarbons decomposed within 2 weeks, using either cultivation method. In the second week small amounts of substances that cannot be easily decomposed by microorganisms, probably cyclohexans or isoparaffins, still remained, but these diminished with the passage of time.

Figure 3-7 presents patterns of decomposition of polycyclic aromatic hydrocarbons.

As the figure shows, in the land farm method, nearly all the polycyclic aromatic hydrocarbons up to 3 rings decompose by the 20th week irrespective of structure. In the course of this change, from the second week, when the saturated hydrocarbons decomposed, decomposition progressed sequentially beginning with those hydrocarbons having the smallest number of rings.

In liquid culture, on the other hand, the microorganisms that can be enriched are limited because the cultivation conditions are aerobic. Figure 3-7 shows that in the decomposition of polycyclic aromatic hydrocarbons, there are declines only in those constituents decomposed by limited types of microorganism.
3.5 Detailed Evaluation of Contamination Status

In research conducted in the last fiscal year, a preliminary investigation was made of soil-contaminated areas using the radar method and the boring method, and the states of contamination in such areas were determined. In the current fiscal year, the radar method was used to see if it is possible to assess contamination in detail in areas of non-uniform oil contamination, and comparison was made between the radar method and boring method in terms of economy.

In the radar method, as a result of measurements taken with a 4 m lattice, it was possible to determine in detail the three-dimensional status of contamination in areas contaminated in a non-uniform manner. Based on these data, the magnitudes of contaminated soils at or above a prescribed concentration could be determined by calculation. It is believed that in this way, the minimum required soil purification process can be implemented and economic soil purification costs can be determined.

In an economic comparison of contamination survey costs, when a detailed evaluation was made by 4 m lattice in the boring method, the following was obtained: excavation cost is ¥10,000/m, the cost of analyzing TPH in soil is ¥15,000/specimen, so that for a measurement area of 45,000 m², the cost is over ¥300 million. In the case of measurement by 4 m lattice, using the radar method, it was estimated that the cost of radar measurement and analysis is ¥10,000/km, while the cost of excavation at 29 locations, plus the cost of analysis of TPH in 87 soil specimens, is less than ¥27 million. In comparison to the boring method, therefore, the cost is reduced to 1/10 or less. Moreover, when boring was conducted at 10 m intervals, the cost came to approximately ¥52 million. It was found that in this case also, the cost with the radar method can be assessed at 1/2 the cost of the boring method. The radar method is a non-destructive approach; it does not cause damage to the soil from excavations, etc., and the states of contamination obtained can be rendered continuously in three-dimensional detail. In the boring method, because measurements are taken at points, a certain degree of estimation is required in determining contamination states, especially when the contamination has not been uniform.

In consideration of such things as economy in the cost of investigation and level of detail in the information obtained, it has been concluded that the radar method is superior for evaluating contaminated areas.

4. Synopsis

4.1 Development of Microorganism Enrichment Technology

Using a liquid circulatory column enrichment unit, fundamental research was conducted for enriching, at good efficiency, microorganisms that decompose heavy oil. It was learned that there is no possibility of drifting in culture liquid circulation, that there is no shortage of nutrient sources during enrichment, that obstructive intermediate metabolites are not produced, and that the pH of circulating culture liquid should be slightly alkaline.
4.2 Small-scale Experiments Using Microorganism Collection Technology

An investigation was made of purification of soil contaminated with heavy oil in relation to changes in enrichment time periods and changes in magnitudes of enriched soil microorganisms added as strains in the small-scale land farm method. In comparison to the conventional method, purification time period, including enrichment time period, could be shortened to 1/3 or less. It was also found that heavy polycyclic aromatic hydrocarbons, which are more difficult to decompose, break down more readily in the land farm method than in the conventional method.

In research conducted this fiscal year, however, because there are such large volumes of enriched soil additives, the focus is on ways to reduce additives still more in the future.

4.3 Determination of Contaminated Soil Status in Detail

The boring method and radar method were used in investigations to determine states of contamination in contaminated soil. It was found that the radar method is superior in terms of both measuring cost economy and determination of details in three-dimensional fashion.

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