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Isolation Of n-Paraffin And C-Heavy Oil-Degrading Bacteria

Joy M. SORROSA[1]
[1]Assistant Professor, Natural Sciences Department, Davao Oriental State College of Science and Technology, Mati, Davao Oriental

Abstract

The isolation and identification of n-paraffin and C-heavy oil-degrading bacteria from five Japanese coastal areas were conducted in 1995. Eight isolates of n-paraffin degrading bacteria were selected from a total of seventy-five which showed well growth in n-paraffin medium. Rate of degradation was analyzed by gas chromatography. Short-chain n-paraffin (CD_{13}, C_{14} and C_{15}) were degraded more effectively than the long chain n-paraffin (C_{16}, C_{17} and C_{18}. Isolates NIWI and N16 have the highest degrading capacity. Degradation rate of n-paraffin increased with an increase of temperature from 10 to 30^0C. Four stage developments with stearyl alcohol as internal reference were found to effectively separate the C-heavy oil components (saturates, aromatics, resins and asphaltene). Degrading capability of the eight selected isolates from 125 isolates of Cheavy oil-degrading bacteria was determined by TLC/FID. Isolate GR211 has the highest degradation activity. Two isolates of n-paraffin degrading bacteria were identified as *Bacillus* sp. (NJWI) and *Micrococcus* sp. (N16)- *Vibrio* sp. (GR211), *Moraxella* sp. (HI l) and *Flavobacterium* sp. (IS3) were identified as C-heavy oil-degrading bacteria.

Keywords: hydrocarbon, degradation, bioremediation, Japanese coastal areas

Introduction

Hydrocarbons are organic compounds that are insoluble in water and are common pollutants in nature associated with the production and utilization of petroleum resources. Industrialization of many countries worldwide lead to an increasing dependency on fossil hydrocarbons and consequently increase requirements for shipping and piping vast amounts of these compounds (Ahearn, 1973). Hydrocarbon-degrading bacteria are most abundant in coastal water and mud where oil pollution is chronic (ZoBell, 1969). Higashihara and Sato (1985) mentioned that these hydrocarbon-degrading bacteria of various genera were widely distributed in pelagic areas. Petroleum pollutants entering the marine environment through spills resulting from transportation and offshore activities pose health hazards to humans and potential damage to the marine environment (Kura, 1995). It was estimated that over 10 million metric tons of petroleum contaminate the world's oceans per year. Booming and sorption could physically recover the contaminating petroleum pollutants. However,

most petroleum pollutants de$' physical collection and are subjected to biodegradation process by natural microbial population in marine environments (Higashihara el al., 1995). Hydrocarbon decomposition in nature involves interactions of complex, physical, chemical and biological processes, the rates of which are interdependent (Ahearn, 1973). Therefore, studies on the distribution and degradation activity of hydrocarbondegrading bacteria in the sea are very important in relation to the capacity of the sea for self-purification and to the development of bioremediation techniques (Leahy and Colwell, 1990; Atlas and Bartha, 1992; Hoff, 1993; Higashihara et al., 1995). In this study, isolation of n-paraffin and C-heavy oil degrading bacteria from various Japanese coastal areas and the taxonomic study were conducted. The estimation rate of degradation by isolated isolates, as well as environmental factors affecting the degradation were also determined.

Materials and Methods

Isolation of n-paraffin and C-heavy oil-degrading bacteria. Seawater and sediment samples were collected from different coastal areas of Japan such as Ibaraki Prefecture, Kochi Prefecture, Takamatsu City, Kobe City and Hiroshima Prefecture.

Seawater samples were filtered and filters were transferred to Erlenmeyer flasks containing 100 ml of Natural Seawater Medium (NSW, Table 1) for isolation and cultivation of marine n-paraffin and C-heavy oil-degrading bacteria. About I gram of each sediment sample was directly transferred to the same medium.

Table 1.The composition of NSW (Higashihara *et al*., 1978)

Components	Amount
Hydrocarbons	0.1~0.5%*
NH_4NO_3	1.0 g
K_2HPO_4	0.02g
Ferric Citrate	0.02 g
Yeast Extract (Difco)	0.5g
Distilled water	200 ml
Aged seawater	800 ml
pH	7.8

*N-alkanes (C_{10}-C_{18} equal volume mixture) 0.5%(v/v); C-heavy oil 0.5%(w/v)

n-Paraffin and C-heavy oil-degrading bacteria were isolated by enrichment and plate culture method. The hydrocarbons used in the study were n-paraffin and C-heavy oil.

Cultivation for assay of bacterial growth and degradation rate. L-test tubes containing 10 ml of NSW were sterilized at 121^0C for 15 minutes. The selected isolates were inoculated to the tubes with 3 replicates and 3 other hydrocarbon-free media were used as the control. Test cultures were incubated on a reciprocal shaker at 20^0C for 14 days.

Daily monitoring of the bacterial growth was done and expressed as optical density (OD) and measured directly at 660 nm using digital turbidimeter.

Analysis of hydrocarbon degradation. Hydrocarbon degradation was calculated on

the basis of residual hydrocarbon in sterile control that was run in the same condition.

n-Paraffin. Residual hydrocarbon was extracted by adding 10 ml of n-hexane. The whole culture was shaken and centrifuged to separate the cells and supematant. Analysis was done by gas chromatography (GC) fitted with a flame ionization detector (FID) with n-C_{20} as internal standard.

C-heavy oil. The cultures were shaken and centrifuged with 10 ml of solvent that was a mixture of n-hexane and toluene (6:4 ratio) to extract the residual hydrocarbons. Solvent was evaporated and the residue was dissolved by choloroform then I ml of stearyl alcohol was added.

A rapid method for compositional analysis by thin layer chromatography (TLC) with FID was employed with four stage development. Figure 1 shows the analytical method of C-heavy oil.

Taxonomic characteristics. Morphological and cultural characteristics of the five selected isolates were identified according to the scheme of Ezura (1990).

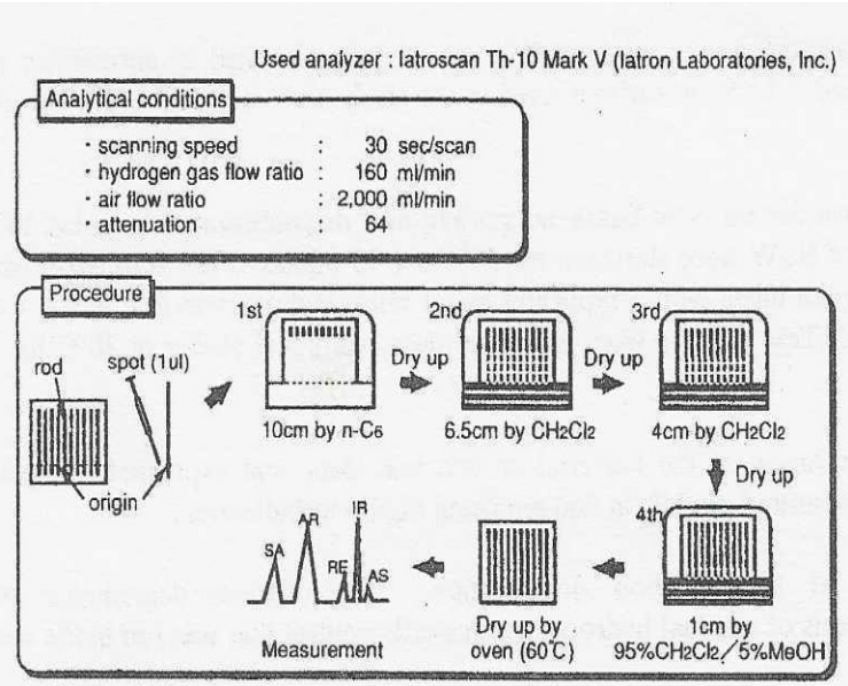

Fig. 1. Analytical method of C-heavy oil

Results and Discussion

There were about 200 isolates from seawater and sediment samples which grew in media containing hydrocarbons such as n-paraffin (NP) and C-heavy oil (HO) as the sole carbon sources (Table 2).

Sixteen isolates found to possess high degradation activity of NP and HO were selected for further study. It is thus suggested that these play a major role in the process of natural self-purification of the seawater from hydrocarbons in these environments (Higashihara et al., 1995).

Growth of isolates of n-paraffin degrading bacteria. There were 8 isolates selected that showed high degrading capacity of the n-paraffin. Bacterial growth of these isolates was measured daily and expressed as optical density (OD).

Results showed that isolate N16 has the highest growth particularly in the first 4 days of incubation period but slowly decreased in the next few days (Figure 2). This is due to the fact that the bacteria adhered to the sides of the tube rendering a lower OD. The second isolate that showed a good growth was NIW1.

It is concluded that these 2 isolates contributed and played a vital role in the overall degradation of n-parafiin in these environments.

Table 2. Hydrocarbon-degrading bacteria isolated from five Japanese coastal areas, 1995

Sampling Location	Sample Code	Isolation Source[1]	Carbon source[2]		Number of Isolates
			NP	HO	
Ibaraki Prefecture	IIW	SW	8	11	19
	IOW	SW	7	4	11
	NIW	SW	7	0	7
	IS	SS	10	2	12
	NS	SS	7	10	17
Sub-total			39	27	66
Kochi Prefecture	MD1	DW		9	9
	MD2	DW		6	6
	MMI	SW		7	7
	MP	SW		7	7
	MS	SW		6	6
	KI			7	7
Sub-total				42	42
Takamatsu City	GR	SW		6	6
	TI	SW		13	13
	TO	SW		4	4
Sub-total				23	23
Kobe City	EK	SW		9	9
Sub-total				9	9
Hiroshima Prefecture	KR	SW	10	3	13
	AO	SW	5	4	9
	OS	SW	8	3	11
	HI	SW	4	5	9
	KS	SW	3	5	8
	NI	SW	6	4	10
Sub-total			36	24	60
Grand Total			75	125	200

[1] SW: Surface sea water, SS: Surface sediment, DW: Deep sea water (Depth 320m)
[2] NP: n-Paraffin (C_{10}~C_{18}) HO: C-heavy oil

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n-Paraffin degrading activity of isolates. Table 3 shows the degrading activity of the 8 selected isolates. Isolate N16 has the highest degradation activity followed by isolate NIW1.

The result showed a good correlation with that of the bacterial growth of these isolates. Data suggest that the hydrocarbon-degrading potential of the bacteria is influenced by their ability to grow and utilize the hydrocarbon as their source of carbon. Higashihara et al., (1995) found that one of the factors affecting the rate at which polluting oils are degraded is the number of microbes present.

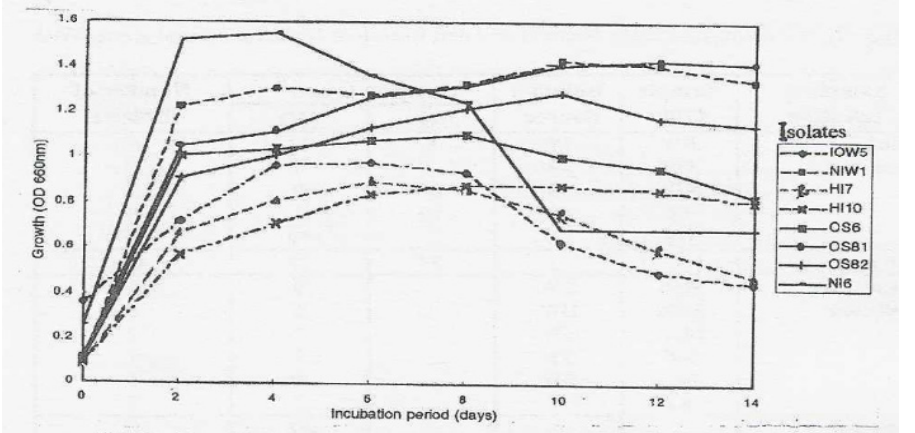

Fig. 2. Growth of isolates in medium containing n-Paraffin

Table 3. n-Paraffin degradation rate of selected isolates from five Japanese coastal areas

Isolate	n-Paraffin(%)					
	C_{13}	C_{14}	C_{15}	C_{16}	C_{17}	C_{18}
IOW5	100	100	100	75.94	63.64	58.25
NIW1	100	100	100	75.62	71.65	65.32
HI7	100	100	61.81	56.88	43.64	28.28
HI10	100	100	100	67.19	52.73	30.98
OS6	100	56.05	51.18	45.94	34.81	25.92
OS8(1)	100	35.03	27.17	24.69	19.74	20.54
OS8(2)	28.44	28.02	22.83	19.06	10.39	0
NI6	100	100	66.93	100	100	48.48

Figure 3 shows that as the temperature increases there is also a corresponding increase in the degradation activity of the 2 isolates. Isolate N16 degraded about 79.5% of the total amount of n-paraffin in the medium at 30^0C, 54% at 20^0C and 29.6% at I OOC. These indicate that temperature affects the degradation activity of these 2 isolates. Most species are most active in the mesothermic range of 20^0C to 35 ^{0}C (ZoBell, 1973). The rate at which oil is degraded is slower at lower temperature.

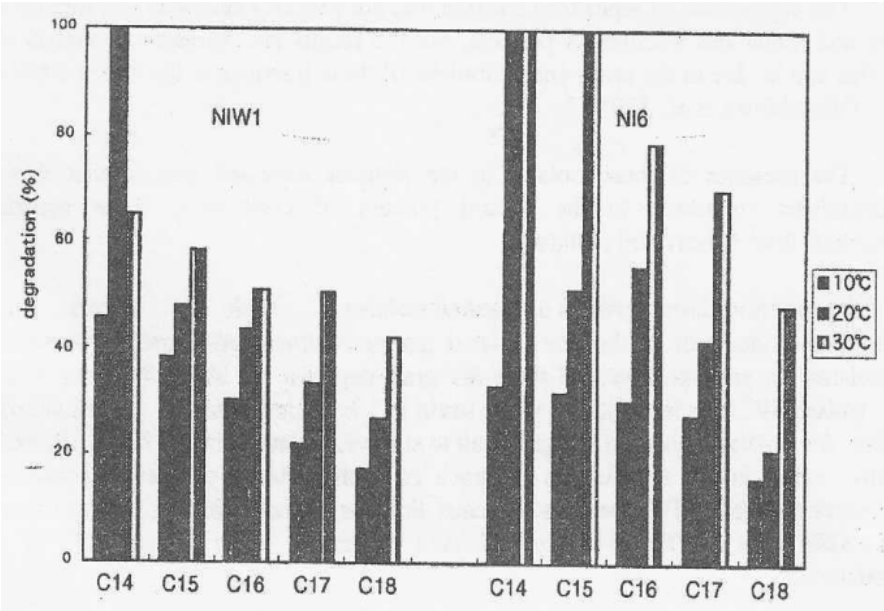

Fig. 3. Effect of temperature on degradation rate of n-Paraffin

C-heavy oil degradation by selected isolates. Better separation of the peaks of the C-heavy oil components was obtained by using four development systems. Stearyl alcohol was chosen as the intemal reference for the analysis because it is solid at room temperature, readily dissolves in choloroform, and was dissolved into separate sharp peaks by the development system used in this study:

Figure 4 shows the degradation rate of the 8 selected isolates. Isolate GR211 exhibited the highest degrading activity with a total degradation of 30%. It was further observed that the degradation of resin component is high at about 38%. The second isolate with a high percentage of degradation was isolate T131 followed by isolate GR212 which degraded the total C-heavy oil component at about 16%.

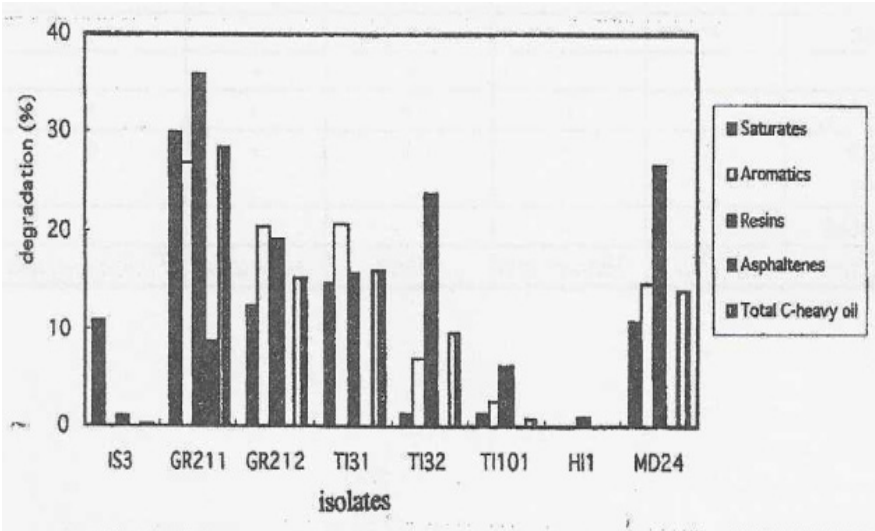

Fig. 4. C-heavy oil degradation rate of isolates

The degradation of asphaltene fraction was not properly detected. Measurement of resin and asphaltene fractions is possible, but the results are subject to analytical error. This can be due to the lower concentrations of these fractions in the hydrocarbons studied (Higashihara et al., 1995).

The presence of these isolates in the samples collected suggest that these microorganisms contribute to the natural process of cleaning-up these marine environments from C-heavy oil pollutants.

Taxonomic characteristics of selected isolates. Table 4 shows the taxonomic characteristics of the five isolated marine hydrocarbon-degrading bacteria. Two isolates are gram-positive and three are gram-negative but all of them are non-motile. Isolate GR211 is fermentative while strain HI 1 is oxidative and all the rest shows no action. All isolates require an inorganic salt to survive. Isolates N16 and NIWI showed a positive result in the test for the presence of spores. Based on these taxonomic characteristics, isolate NIWI belongs to genus Bacillus, isolate N16 is a Micrococcus, isolate GR211 is a Vibrio, isolate HII is a Moraxella while isolate IS3 is a Flavobacterium.

It is suggested that these isolates be studied further for possible inclusion in bioremediation techniques to clean-up marine environments contaminated by petroleum pollutants.

Table 4. Taxonomic characteristics of selected isolates

Characteristic	Degrading Bacteria				
	n-Paraffin		C-Heavy Oil		
	NIW1	NI6	GR211	HI1	IS3
Gram stain	+	+	-	-	-
O-F test	No action	No action	Fermentative	Oxidative	No action
Motility	-	-	-	-	-
Pigmentation	+	-	+	-	+
Oxidase	-	-	+	+	-
Catalase	+	+	+	+	+
Inorganic salt requirements	+	+	+	+	+
Spore produced	+	+			
Identified genus	*Bacillus*	*Micrococcus*	*Vibrio*	*Moraxella*	*Flavobacterium*

Conclusion

Several factors affect the rate of degradation of n-paraffin such as the carbon number of n-paraffin (short chain n-paraffin are more effectively degraded than the long chain n-paraffin) and temperature (degradation rate increased with the increase in temperature). Hydrocarbon-degrading potential of the bacteria is influenced by their ability to grow and utilize the hydrocarbon as their source of carbon.

Compositional analysis of C-heavy oil could be accurately performed by four stage developments using n-hexane with a development height of 10 cm, dichloromethane of 6.5 cm, another dichloromethane of 4 cm and 95% dichloromethane and 5% methanol of 1 cm with stearyl alcohol as internal reference.

Bacillus sp. (isolate NIW1), *Micrococcus* sp. (isolate N16), *Vibrio* sp. (isolate GR211), *Moruella* sp. (isolate HI1) and *Flavobacterium* sp. (isolate IS3) are deemed useful for bioremediation techniques to clean-up marine environments contaminated by petroleum pollutants.

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