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Biological Removal of Pyridine in Heavy Oil by *Rhodococcus* sp. KCTC 3218

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The removal of organic nitrogen compounds present in crude petroleum and shale oils poses a challenging problem in petroleum industries. The deleterious effect of nitrogen compounds on cracking catalysts and the indication that they contribute to gum formation in gasolines are some of these aspects. Pyridine, a representative nitrogen compound in heavy oil - was degraded by *Rhodococcus* sp. KCTC 3218 in a water-heavy oil two-phase system. The pyridine degradation rate was affected by the presence of hydrocarbons such as n-hexadecane. This microorganism formed flocs which could be a barrier to mass transfer between the cells in flocs and the pyridine dissolved in water. This problem could be overcome by the addition of a surfactant such as Triton X-100. The ratio of water to heavy oil was important to separate the heavy oil phase from the water phase after treating the heavy oil. The culture medium was emulsified by a sort of biosurfactant secreted by this microorganism. The emulsified oil phase returned to its natural state when the ratio of water to heavy oil was 1.5. Above this ratio, the emulsified oil phase remained an emulsion after decantation. Pyridine in heavy oil was completely degraded in 15 hr at this water to heavy oil ratio of 1.5 when the concentration of pyridine in heavy oil was 700 ppm and the cell concentration was 0.32 g DCW/L.

Key words: pyridine degradation, heavy oil, *Rhodococcus* sp., two-phase system

INTRODUCTION

Recently, the microbial degradation of nitrogen compounds from fossil fuels has been drawing increasing attention because of the contribution these contaminants make towards the formation of nitrogen oxides and hence towards air pollution and acid rain. They also contribute to catalyst poisoning during the refining of crude oil, thus reducing process yields [1].

In order to alleviate these problems, microorganisms and biological processes are needed for the efficient and economical removal of aromatic nitrogen compounds. Current researches on the microbial degradation of nitrogen compounds mainly focus on the development of microorganisms and identification of pathways [2,3]. However, little has been done in the development of a biological process due to the limitation imposed by the heterogeneous process system which comprises an oil fraction (organic phase) and a biocatalyst (aqueous phase), despite the need for the development of a process for the degradation of nitrogen compounds.

The reactivity of heterocyclic nitrogen compounds such as pyridine, quinoline, and acridine is very low. Among these compounds, pyridine is the most important compound because the other compounds containing the pyridine ring have low reactivity. Pyridine is readily degraded in soil and a number of soil bacteria are capable of growth on pyridine as the sole source of carbon [4].

In this study, we employed a water-heavy oil two-phase system, which can be used to degrade high concentrations of pyridine in heavy oil by partitioning the inhibitory substrate between the water and heavy oil phases at sub-inhibitory levels, and investigated the possibility of pyridine removal from heavy oil using this system.

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MATERIALS AND METHODS

Microorganism

The microorganism used in this study was *Rhodococcus* sp. KCTC 3218 obtained from the Korean Collection for Type Cultures (KCTC).

Media and Culture Conditions

The composition of the minimal salt medium (MSM) used is as follows. K_2HPO_4 - 0.9 g/L; KH_2PO_4 - 0.54 g/L; KCl - 0.25 g/L; $MgSO_4$ - 0.25 g/L, 1 mL trace element solution in 1 liter distilled water. KCl and $MgSO_4$ were added after autoclaving. The trace element solution contained per 1 L : $FeSO_4 \cdot 7H_2O$ – 2 100 mg; $CoCl_2 \cdot 6H_2O$ - 250 mg; $NiCl_2 \cdot 6H_2O$ - 24 mg; $CuCl_2$ - 5 mg; $MnCl_2 \cdot 4H_2O$ - 100 mg; $ZnCl_2$ - 144 mg; H_3BO_3 - 30 mg; $Na_2MoO_4 \cdot 2H_2O$ - 36 mg; EDTA ($C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$) - 5769.1 mg. 100 mL of the cell culture media was dispensed into 250-mL Erlenmeyer flasks. All incubations were carried out on a shaking incubator at 250 rpm and 32°C. Pyridine (Junsei, Tokyo, Japan) and other hydrocarbon compounds (Sigma

Chemical Co., St. Louis, MO, U.S.A.) used in this study were of reagent grade. Heavy oil was obtained from Sunkyung R & D center, Taejon, Korea.

Assay

For the analysis of pyridine, centrifuged samples were analyzed by isocratic reverse phase HPLC. A radial compressed INERTSIL ODS-3 resolve C18 column (GL Sciences Inc., Tokyo, Japan) in combination with a Hitachi L-4200 UV-VIS detector was used. The Mobile phase was composed of 900 mL HPLC grade methanol and 2 100 mL distilled water. The injection volume of the samples was 10 μL. The flow rate was 1 mL/min. The column temperature was maintained at 40°C. Pyridine has maximum absorbance at 254 nm and the amount of pyridine in the culture supernatant was determined at this wavelength. Optical density of the culture broth sample was measured at a wavelength of 540 nm by a UV/Visible spectrophotometer. The ratio of dry cell weight (g/L) to optical density was 0.306.

The glucose concentration was determined by an enzymatic method (GOD-POD method) using Glucose-E kits (Yeongdong Pharmaceutical Corp., Seoul, Korea). Ammonia concentration was determined by the Nessler method [5].

The cell hydrophobicity was determined by the method of Rosenberg *et al.* [6].

RESULTS AND DISCUSSION

Growth of *Rhodococcus* sp. KCTC 3218 on Different Hydrocarbons

The growth of *Rhodococcus* sp. KCTC 3218 on nitrogen-containing compounds and hydrocarbons as sole carbon source was observed (Table 1). Short chain *n*-alkanes (below C-12) were not degraded. One of the reasons guessed was the toxicity of these compounds. In fact, alkanes of chain length from C-10 to C-20 are optimal for biodegradation of hydrocarbons [7]. This organism was rather selective in its choice of heterocyclic growth substrates and nitrogen compounds such as aniline, pyrrole and indole were not utilized for growth. Quinoline which has a pyridine ring was degraded when used as the sole carbon source but the growth was low because of its low solubility and toxicity. The ability of *Rhodococcus* sp. KCTC 3218 to use quinoline was consistent with its ability to use pyridine as the sole carbon and nitrogen source since a single nitrogen atom is present in the pyridine ring. In the water-heavy oil system, *n*-alkanes can play an important role in transporting compounds with low solubility such as quinoline and dibenzothiophene to the microorganism.

Typical results of cultivation by *Rhodococcus* sp. KCTC 3218 using 1 000 ppm pyridine as a substrate is shown in Fig. 1. Only 31% of the carbon in the pyridine ring was converted into cell mass and about 56% of the nitrogen in the pyridine ring was converted into free ammonia and released to the culture broth. Thus, almost all the nitrogen in the pyridine ring was converted to free ammonia and cell mass thus demonstrating that pyridine was completely degraded.

Table 1. Growth of *Rhodococcus* sp. KCTC 3218 on different hydrocarbons

Hydrocarbon	Growth	Hydrocarbon	Growth
n-Pentane	-	Isobutyl alcohol	-
n-Hexane	-	Benzene	-
n-Heptane	-	Phenol	+
n-Octane	-	Cyclohexanol	++
n-Dodecane	-	*m*-Cresol	++
n-Tetradecane	+	Toluene	-
n-Hexadecane	++	Aniline	-
1-Propanol	++	Pyrrole	-
2-Propanol	-	Indole	-
Methanol	-	Pyridine	++
Ethanol	++	Quinoline	+

(-: no growth, +: weak growth, ++: strong growth)

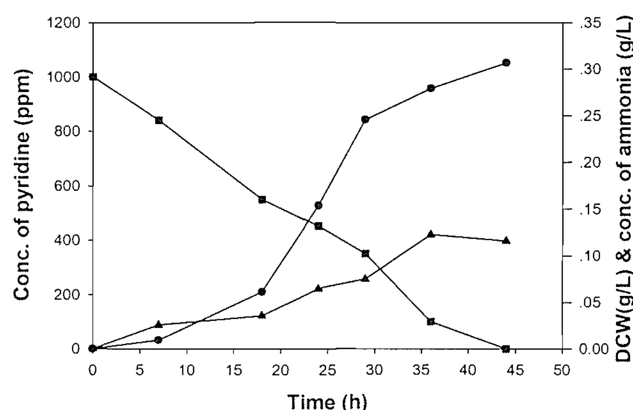

Fig. 1. Concentration profiles of pyridine, ammonia, and cell during the degradation of pyridine (■: pyridine, ●: cell, ▲: ammonia).

Effect of *n*-Hexadecane on the Degradation of Pyridine

The effect of added *n*-hexadecane on pyridine degradation by *Rhodococcus* sp. KCTC 3218 in actual heavy oil was investigated. The degradation rate of pyridine did not decrease significantly due to the presence of *n*-hexadecane in the range of 0.1-0.5% (v/v), but the degradation rate of pyridine decreased at *n*-hexadecane concentrations above 1% (v/v) (Fig. 2(a)). Microscopic observation showed that most of the cells attached to large oil drops and formed flocs as had been reported by Mimura *et al.* [8]. *Rhodococcus* sp. KCTC 3218 produced microbial flocculants as had been reported by Kurane *et al.* [9]. No extracellular flocculant was produced by this microorganism when grown on glucose, LB, and pyridine. The formation of flocs decreases the number of freely suspended cells in the culture broth. So the chance of cells to contact the pyridine solubilized in the medium decreases and the degradation rate of pyridine decreases too. When the initial cell density is high, the degradation rate of pyridine was not affected by the addition of 10% (v/v) *n*-hexadecane to MSM containing 1 000 ppm pyridine but was affected by the addition of 10% (v/v) heavy oil (Fig. 2(b), (c)). One probable reason is that heavy oil, in fact, is a

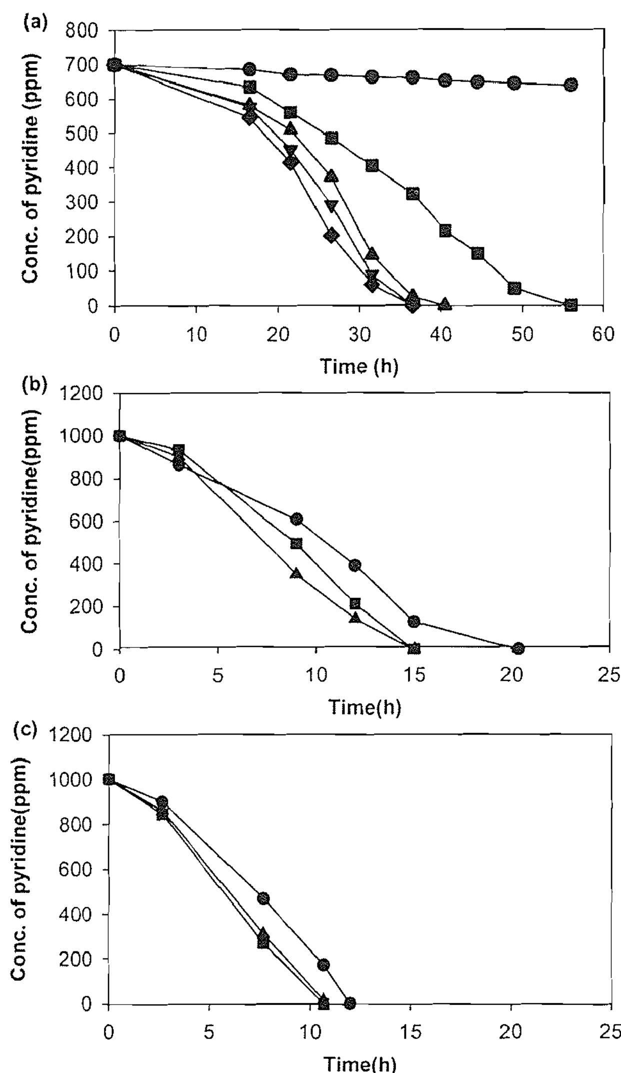

Fig. 2. Effect of n-hexadecane and heavy oil on pyridine degradation; (a) Effect of n-hexadecane(n-C16) on the degradation rate of pyridine (Initial cell concentration: 0.031 g DCW/L, ●: control, ◆: 700 ppm pyridine, ▼: 700 ppm pyridine and 0.3%(v/v) n-C16, ▲: 700 ppm pyridine and 0.5%(v/v) n-C16, ■: 700 ppm pyridine and 3%(v/v) n-C16). (b), (c) Effect of heavy oil and n-hexadecane on the degradation rate of pyridine for different initial cell concentrations (Initial cell concentration: b: 0.12 and c: 0.6 g DCW/L; ●: 1 000 ppm pyridine and 10% (v/v) heavy oil, ■: 1 000 ppm pyridine and 10% (v/v) n-C16, ▲: 1 000 ppm pyridine).

complex mixture of different hydrocarbons which may in turn affect the degradation rate of pyridine.

Hydrophobic Characteristics of *Rhodococcus* sp. KCTC 3218

The hydrophobicity of *Rhodococcus* sp. KCTC 3218 depended on the substrate in which the cell was grown. In fact, while pyridine was used as a substrate, the microorganism did not exhibit hydrophobic characterisitics, but it exhibited hydrophobic characteristics in the presence of n-hexadecane and heavy oil (Fig. 3). The hydrophobic nature of the outermost surface of various microbial cells has been reported [10]. As for the degradation of quinoline

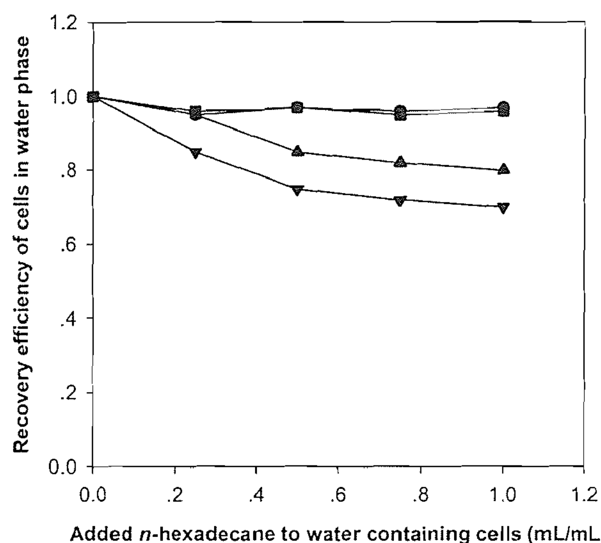

Fig. 3. Hydrophobicity of *Rhodococcus* sp. KCTC 3218 (●: Glucose grown cells, ■: Pyridine grown cells, ▲: n-hexadecane-grown cells, ▼: Heavy oil-grown cells).

which has a low solubility, the hydrophobic factor characterized by the capability of the bacteria to adhere to the hydrocarbon phase may be important. *Rhodococcus* sp. KCTC 3218 may use insoluble compounds through direct contact because of its hydrophobicity [11].

Effect of Flocs on Pyridine Degradation Rate

Rhodococcus sp. KCTC 3218 formed flocs in the presence of n-hexadecane and heavy oil. The cause of these flocs appeared to be linked to cell hydrophobicity and hydrocarbon droplets. These flocs consist of an agglomeration of cells, hydrocarbon droplets, and air bubbles bound typically together. Once the cells are present in flocs, contact between cells and pyridine or the transfer of dissolved oxygen may become rate limiting in the center of these flocs, analogous to the situation observed in mycelial pellets. We used Triton X-100 to prevent adherence of cells to n-hexadecane. Addition of 0.7 ppm Triton X-100 to MSM containing 700 ppm pyridine and 1% (v/v) n-hexadecane decreased the extent of formation of flocs and increased the degradation rate of pyridine (data not shown). This indicates that floc formation could decrease the degradation rate of pyridine in the presence of n-hexadecane.

Biodegradation of Pyridine in Heavy Oil Using Water-Heavy Oil Two-Phase System

Washed cells grown on MSM and 1 000 ppm pyridine for 30 hr were used in a water-heavy oil two-phase system to treat the pyridine in heavy oil containing 1 000 ppm pyridine. The total working volume was fixed as 100 mL and the ratio of water to heavy oil was varied from 9 to 0.43. The pyridine in heavy oil was completely removed by *Rhodococcus* sp. KCTC 3218. The rate of pyridine degradation decreased as the ratio of water phase to heavy oil phase decreased from 9 to 0.43 (Fig. 4). The low rate

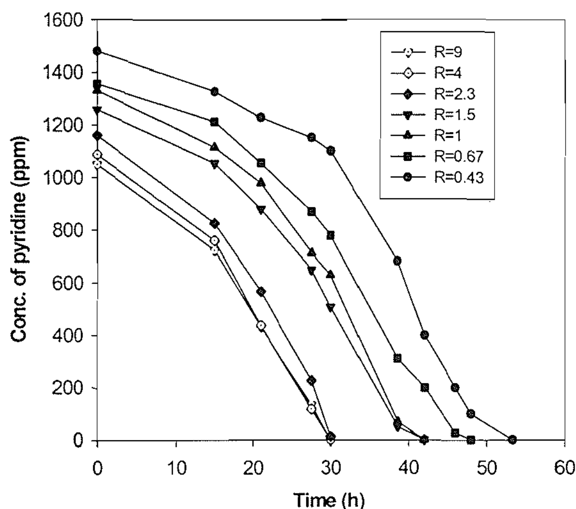

Fig. 4. Degradation of pyridine in heavy oil containing 1 000 ppm pyridine using water heavy oil two-phase system (R: the ratio of water to heavy oil).

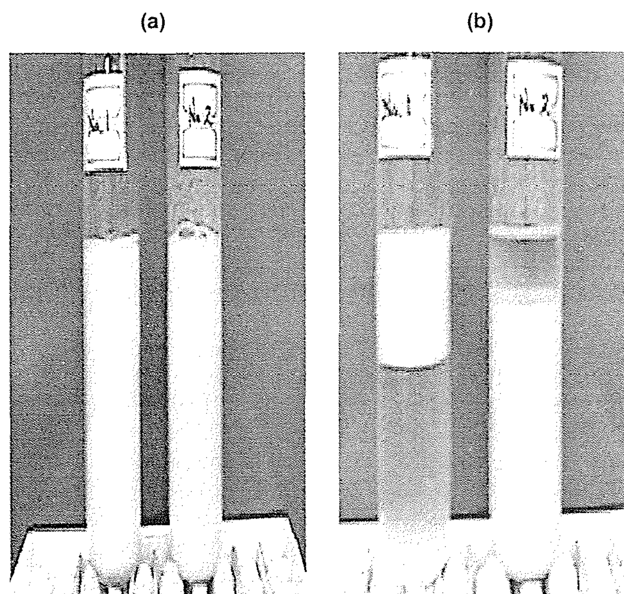

Fig. 5. The culture broth emulsified by *Rhodococcus* sp. KCTC 3218 ((a): before decantation and (b): after decantation, in each photo: left: the ratio of water to heavy oil – 2.5 and right: the ratio of water to heavy oil – 1.5).

of pyridine degradation for low values of the water to heavy ratio oil may be attributed to factors such as the initial concentration of pyridine, oxygen transfer, and toxicity. The ratio of water phase to heavy oil phase is important in the recovery of the heavy oil phase after treating the heavy oil. Emulsification was observed in the water-heavy oil system during batch culture. It has been noted that the hydrocarbon added to the fermentation broth was emulsified by a phenomenon other than simple mechanical agitation [13]. When the ratio of water phase to heavy oil phase exceeded 1.5, the emulsified oil phase did not return to its natural state and the water phase was clear. But the emulsified oil phase returned to its natural state and the water phase remained an emulsion at the ratio of 1.5 and below

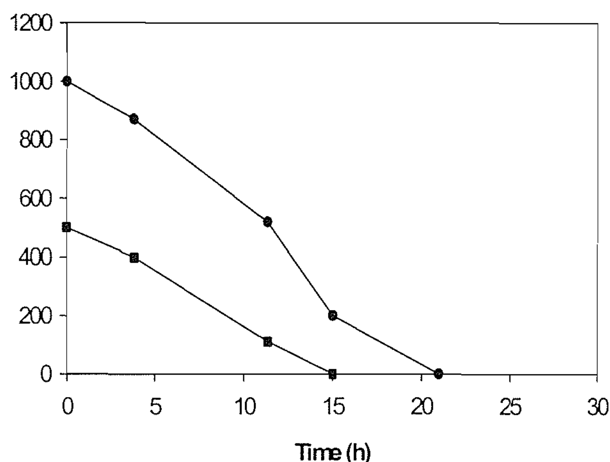

Fig. 6. Degradation of pyridine in heavy oil when the ratio of water to heavy oil is 1.5 (Initial cell density 0.32 g/L: ■: heavy oil containing 700 ppm pyridine, ●: heavy oil containing 1 500 ppm pyridine).

it (Fig. 5). Therefore, a water to heavy oil ratio of 1.5 was optimal for the recovery of heavy oil after biological treatment. Pyridine in heavy oil was completely degraded in 15 hr at this water to heavy oil ratio when the concentration of pyridine in heavy oil was 700 ppm and the cell concentration was 0.32 g DCW/L (Fig. 6).

CONCLUSION

The water-heavy oil two-phase system has the advantage of being self-regulating in terms of pyridine delivery to the water phase in response to the rate of consumption by the cells and it eliminates the problem of substrate inhibition commonly observed at high concentrations in a batch system. In the water-heavy oil two-phase system, pyridine diffuses towards the water phase in which the microorganism dwells because of its miscibility in water. This system is considered ideal for the removal of pyridine in heavy oil because of the good miscibility of pyridine in water.

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