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Mixed Culture of *Mycotorula japonia* and *Pseudomonas oleovorans* on Two Hydrocarbons†

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A mixed culture system of *Mycotorula japonica* IAM 4185 and *Pseudomonas oleovorans* IFO 12570 was established on two carbon sources, *n*-tetradecane and phenol, as a model for the treatment of petroleum sewerage. Characteristics of this system were investigated in batch and continuous cultures. The two microbes exhibited commensalism with participation of biotin; *P. oleovorans* excreted biotin vitamer, which *M. japonica* required for growth. The former assimilated phenol and the latter mainly *n*-tetradecane in batch culture. In continuous culture, this system was unstable and an oscillatory phenomenon was observed for a long period.

Heterogeneous microbial populations[1] are classified as follows: neutralism, competition, mutualism, commensalism, amensalism, parasitism and predation. In the cases of commensalism and mutualism[3,4] a mixed culture system is essential to support the growth of both microorganisms.

In the previous paper,[2] it was reported that in a mixed culture system of *Mycotorula japonica* IAM 4185 and *Bacillus subtilis* JB 15 on glucose, commensalism was observed; *M. japonica* required biotin as a growth factor and *B. subtilis* excreted biotin vitamer.

Shindala *et al.*[5] reported the mixed culture of *Proteus vulgaris* with *Saccharomyces cerevisiae* in continuous culture. In their system, a chemically defined medium was selected which supported the growth of *Saccharomyces cerevisiae*, but not *P. vulgaris* in pure culture; however, *P. vulgaris* grew in mixed culture with the yeast. The two microbes had a commensal relationship, namely, an essential niacin-like factor, which was produced by the yeast and required by the bacterium, caused the dependence of the bacterium on the growth of the yeast.

Chian and Mateles[6] reported the mixed continuous culture on mixed glucose-lactose or glucose-butyrate media inoculated with river water. They found that at a high dilution rate, pseudomonad dominated the population, whereas at low and moderate dilution rates coliforms were dominant, using natural mixed culture.

In this work, the behavior of a mixed culture was investigated, employing *Mycotorula japonica* and *Pseudomonas oleovorans* which exhibited commensalism and could be established on two carbon sources, *n*-tetradecane and phenol. This commensal system was established as a model for the treatment of multi-substrates in hydrocarbon sewerage. Using this mixed culture system, the characteristics of the mixed culture were investigated.

Materials and Methods

Microorganisms and cultivation methods

Mycotorula japonica IAM 4185 and *Pseudomonas oleovorans* IFO 12570 were employed.

The composition of basal medium was as follows: NH_4NO_3, 1.0; K_2HPO_4, 3.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02; $FeCl_2 \cdot nH_2O$, 0.02; $ZnSO_4 \cdot 7H_2O$, 0.01 and NaCl ,0.1 (g/*l*), in distilled water. As carbon sources, *n*-tetradecane and phenol were used for mixed culture, *n*-tetradecane was 1,000 mg/*l* and phenol 500

† Special contribution to this valedictory issue for Professor G. Terui.

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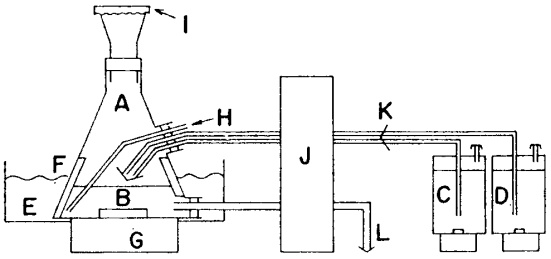

Fig. 1. Flow sheet of continuous culture system.
A: fermentor (1-*l* Erlenmeyer flask, working volume 300 ml), B: 50 mm spine bar, C: storage tank for emulsified *n*-tetradecane, D: storage tank for basal medium and phenol, E: water bath, F: baffle, G: magnetic stirrer, H: sampling tube, I: cotton and gauze, J: pump, K: inlet, L: outlet.

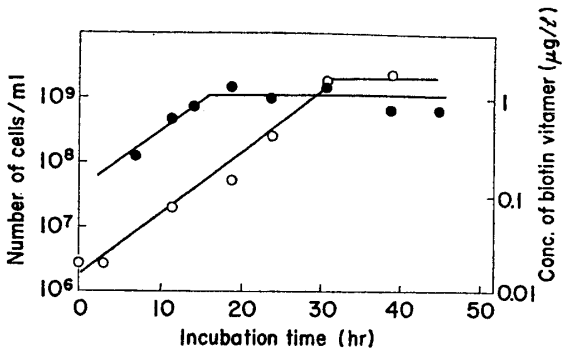

Fig. 2. Biotin production by *P. oleovorans* on 500 mg/*l* phenol in batch culture.
○ Cell, ● Biotin.

mg/*l* in batch and continuous cultures. To examine the toxic effect of phenol on the growth of *M. japonica*, phenol concentrations were varied from 30 to 1,500 mg/*l* ,and on *P. oleovorans*, phenol concentrations were from 10 to 500 mg/*l* in batch culture. 0.2 μg/*l* biotin was added to pure culture of *M. japonica*.

In actual treatment of hydrocarbon sewerage, oil drops are usually separated by an oil separator and after separating, only fine oil drops remain and these oil drops are treated with microbes. Thus, 1,000 mg/*l* *n*-tetradecane was emulsified with a surface active agent, Spane 40, 100 mg/*l* ,by ultrasonicator.

Batch culture was carried out in a 500-ml Erlenmeyer flask with 100 ml medium on a reciprocal shaker. The apparatus used for the continuous culture is shown in Fig. 1. The reactor was a 1,000-ml Erlenmeyer flask with baffles of a working volume of 300 ml. A magnetic stirrer with a 50 mm spin-bar was used for agitation and aeration. Cultivations were carried at 30°C.

Analytical methods The 4-amino antipyrin method (JIS K0102, 1971) was employed for the analysis of phenol. *n*-Tetradecane in the culture broth was extracted with carbontetrachloride and its concentration measured by infrared spectrometry (JIS K0102, 1971).

The bioassay of biotin vitamer was carried out by using *M. japonica* IAM 4185 as test organism.[2]

Numbers of *M. japonica* were counted by haemocytometer and *P. oleovorans* by a bacteria counting chamber, and the cell concentration was measured by optical density at 660 nm. Prior to the measurement of the optical density, *n*-tetradecane in the culture broth was dissolved by a mixed solvent (ethanol and ether whose ratio was 3 to 1).

Results and Discussion

Establishment of mixed culture system

n-Tetradecane and phenol were selected as a multi-substrates of petroleum sewerage. *M. japonica* IAM 4185 requires biotin vitamer for growth[2] and can assimilate hydrocarbons. Thus, a microorganism for mixed culture with *M. japonica* needs to satisfy the following conditions.

(1) The organism must excrete biotin vitamer.

(2) It is desirable that one organism assimilate mainly one substrate and the other organism assimilate mainly another substrate, to allow simplicity of system analysis and stability of the system.

Pseudomonas oleovorans IFO 12570 was selected for these requirements. Figure 2 shows the time courses of the growth and biotin vitamer production of *P. oleovorans*. The rate of biotin vitamer production by *P. oleovorans* was parallel to the growth rate and the production quantity was in excess for the requirements of *M. japonica* as shown in our previous work.[2]

The toxic effects of phenol on the growth of *M. japonica* and *P. oleovorans* were examined. The phenol toxicity scarcely affected the specific growth rate of *M. japonica* at concentrations lower than 1,000 mg/*l* (Fig. 3).

The specific growth rate was decreased slightly with 1,250 mg/*l* phenol. *M. japonica* could not grow on 1,500 mg/*l* phenol even after 100 hr culture. The lag-time of *M. japonica* had a tendency to get longer with the increase of phenol concentration. The growth rate of *P. oleovorans* was not affected by phenol in concentrations lower than 100 mg/*l*, but the growth rate was slightly de-

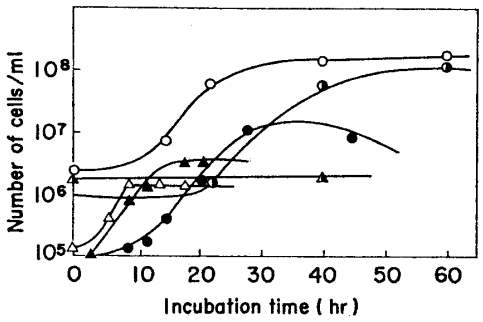

Fig. 3. Toxic effect of phenol on growth of *M. japonica*. 0.2 μg/*l* biotin was added.
Phenol concentration

△	30 mg/*l*	▲	100 mg/*l*
●	500	○	1,000
◑	1,250	◭	1,500

creased on 500 mg/*l* phenol as shown in Fig. 4.

Substrate assimilation and microbial growth in batch culture In the absence of biotin, *M. japonica* in pure culture did not grow, but *M. japonica* in mixed culture with *P. oleovorans* could grow well. A typical mixed culture on the two substrates, *n*-tetradecane and phenol, is shown in Fig. 5. In this case, phenol was assimilated before *n*-tetradecane began to be assimilated. *P. oleovorans* which excreted biotin began to grow prior to *M. japonica* which required biotin. The interaction in the mixed culture was considered to be caused by commensalism.

Next, the assimilation of each substrate was examined. *M. japonica* and *P. oleovorans* were cultured on phenol as a single carbon source. Both microbes can assimilate phenol as shown in Fig. 6. Pure culture of each microorganism was done on *n*-tetradecane.

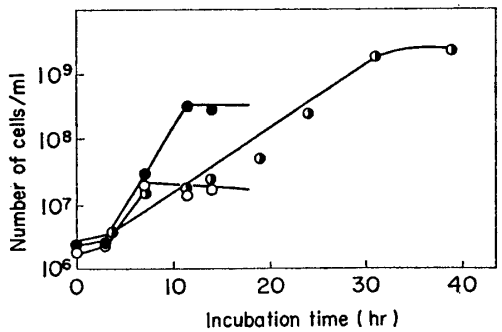

Fig. 4. Toxic effect of phenol on growth of *P. oleovorans*. Phenol concentration:
○ 10, ● 100, ◑ 500 mg/*l*.

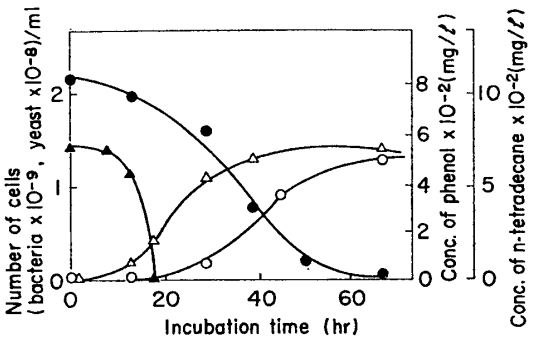

Fig. 5. Mixed batch culture of *P. oleovorans* and *M. japonica* on two carbon sources.
○ *M. japonica*, ● *n*-Tetradecane, △ *P. oleovorans*, ▲ Phenol.

It was obvious from the results in Fig. 7 that *P. oleovorans* could not assimilate *n*-tetradecane. Consequently, it was found, from the above results, that in the case of the mixed culture on the two carbon sources, *P. oleovorans* could assimilate phenol only faster than *M. japonica*, while *M. japonica* could assimilate *n*-tetradecane and phenol.

Substrate assimilation and microbial

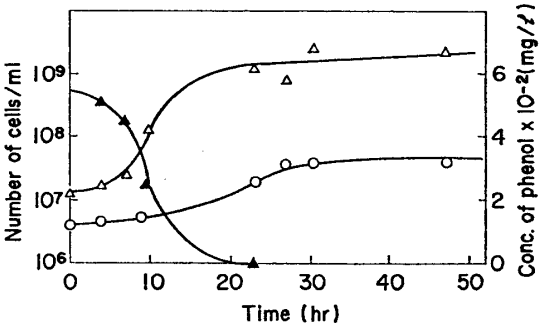

Fig. 6. Mixed batch culture of *P. oleovorans* and *M. japonica* on 500 mg/*l* phenol.
○ *M. japonica*, △ *P. oleovorans*, ▲ Phenol.

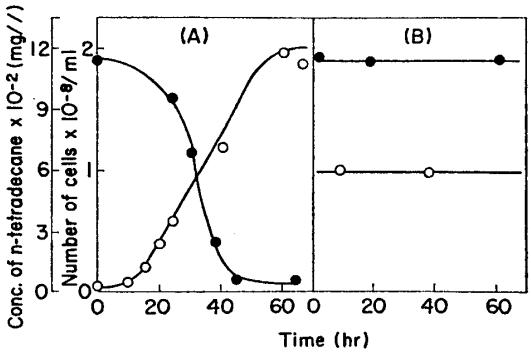

Fig. 7. Pure cultures of *M. japonica* (A. addition of biotin 0.2 μg/*l*) and *P. oleovorans* (B) on *n*-tetradecane in batch culture.
○ Cell, ● *n*-Tetradecane.

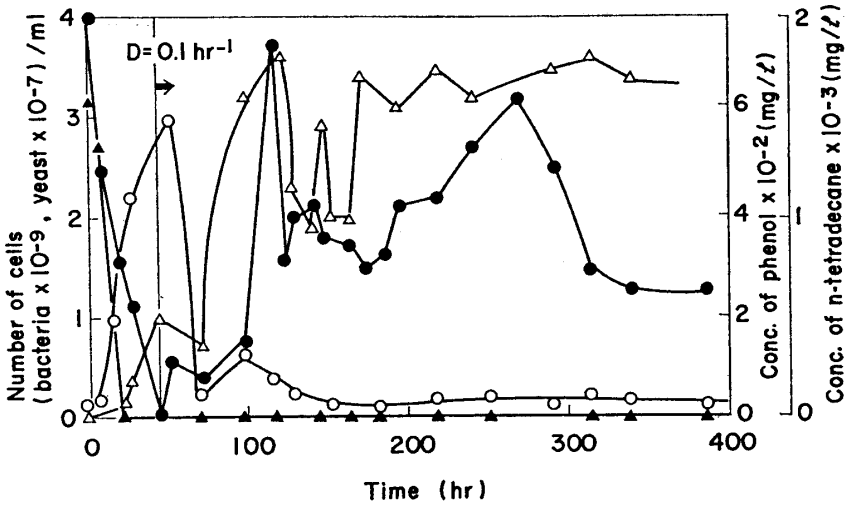

Fig. 8. Mixed continuous culture of *P. oleovorans* and *M. japonica* on two carbon sources. Concentration of inlet *n*-tetradecane was 1,000 mg/*l* and phenol 500 mg/*l* in continuous culture ($D=0.1\ hr^{-1}$). Arrow (→) indicates start of continuous culture.
○ *M. japonica*, ● *n*-Tetradecane, △ *P. oleovorans*, ▲ Phenol.

growth in continuous culture Continuous cultures were carried out according to the results of batch culture. In these experiments, the batch culture was initiated and then switched to a continuous culture. Figure 8 shows the results of a mixed continuous culture of *M. japonica* and *P. oleovorans* at the dilution rate of 0.1 hr^{-1}. The results demonstrate that the populations of both microorganisms showed oscillations during about ten-fold of the mean residence time in the transient phase from batch to continuous culture, and then they reached steady state. The cell concentration of *M. japonica* was kept constant and relatively low. Consequently, the concentration of residual *n*-tetradecane was high. It might be considered from the phenomenon that this apparent steady state was unstable, because the concentration of the rate limiting substrate was

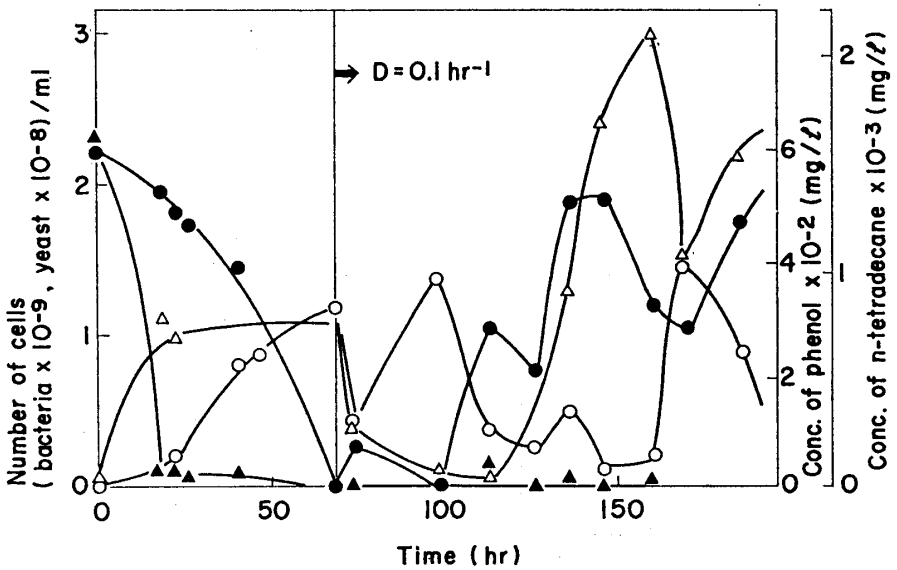

Fig. 9. Mixed continuous culture of *P. oleovorans* and *M. japonica* on two carbon sources. Experimental conditions were the same as Fig. 8. Arrow (→) indicates start of continuous culture.
○ *M. japonica*, △ *n*-Tetradecane, ● *P. oleovorans*, ▲ Phenol.

very high. On the other hand, the concentration of residual phenol was lower than 5 mg/*l*. In order to examine the reproducibility of this oscillatory phenomenon, the same mixed culture was carried out with the same two carbon sources. The results are shown in Fig. 9, which indicate that the populations oscillate during more than ten-fold of the mean residence time. This oscillatory phenomenon is unexpected from pure culture with usual chemostat.

As this oscillation in the mixed culture was expected to continue for a fairly long time, long continuous cultures were carried out. In this case, the dilution rate employed was 0.05 hr^{-1}, because it is considered that continuous culture is more stable at a lower dilution rate. The results are shown in Fig. 10, which indicate that both the microorganisms oscillated not only in the transient phase but also for a longer time and the culture is unstable even at the dilution rate of 0.05 hr^{-1}. This instability in a mixed continuous culture can not be found in a usual pure continuous culture with a chemostat, and it is considered to be a characteristic phenomenon of a mixed culture system based on commensalism on multi-substrates.

Further, a continuous culture was carried at a higher dilution rate i.e. $D=0.15$ hr^{-1}. In this case, *M. japonica* was washed out as shown in Fig. 11. Generally, the microbial affinity for water-insoluble hydrocarbons is lower than for water-soluble substrates. Therefore, the dilution rate has a great effect on cell concentration for the continuous culture on water-insoluble hydrocarbons.

The following causes may be considered for the instability in this continuous mixed culture on the two carbon sources;

(1) commensalism in which the biotin vitamer that *P. oleovorans* excreted was required

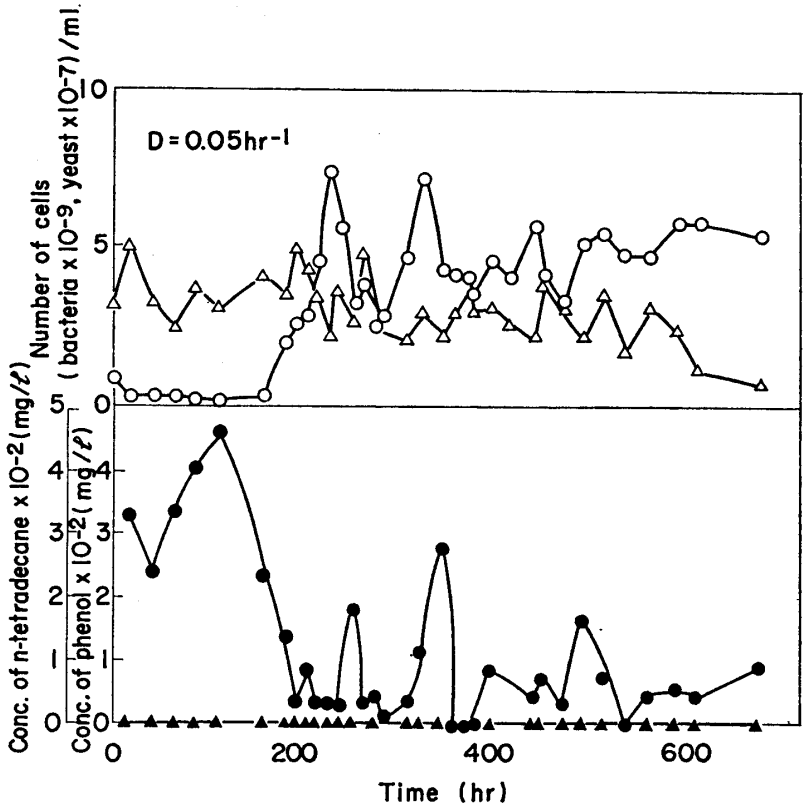

Fig. 10. Mixed continuous culture of *M. japonica* and *P. oleovorans* on two carbon sources.
Experimental conditions were the same as Fig. 8 except that the dilution rate was 0.05 hr^{-1}.
○ *M. japonica*, ● *n*-Tetradecane, △ *P. oleovorans*, ▲ Phenol.

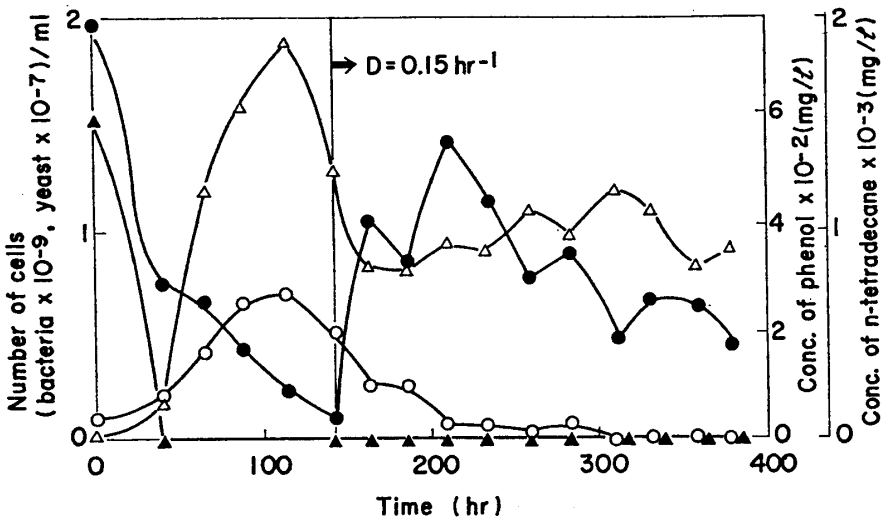

Fig. 11. Mixed continuous culture of *M. japonica* and *P. oleovorans* on two carbon sources. Experimental conditions were the same as Fig. 8 except that the dilution rate was 0.15 hr^{-1}. Arrow (➞) indicates start of continuous culture.
○ *M. japonica*, ● *n*-Tetradecane, △ *P. oleovorans*, ▲ Phenol.

by *M. japonica* for growth,
(2) competitive assimilation of phenol by the two microorganisms,
(3) phenol toxicity toward the growth of *P. oleovorans* or *M. japonica*,
(4) repressive assimilation by *M. japonica* on the two substrates,
(5) low affinity of *M. japonica* for *n*-tetradecane.

In this work, the concentration of phenol in the culture broth was very low i.e. less than 5 mg/*l*, so it is not considered that phenol toxicity is the cause of the oscillatory phenomenon.

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