Front. Biol. China 2007, 2(4): 387–390
DOI 10.1007/s11515-007-0058-7

RESEARCH ARTICLE

Optimized cultivation of highly-efficient degradation bacterial strains and their degradation ability towards pyrene

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Abstract Two bacterial strains, Py1 and Py4, have been tamed and isolated through long cultivation with polycyclic aromatic hydrocarbon—pyrene as the single carbon source. It has been proven that they are both highly-efficient pyrene degrading bacteria and both *Bacillus sp.*. The pyrene degradation ability of separated Py1, Py4 and the consortium of equal Py1 and Py4 was studied in this project. It is shown that pyrene degradation rates were 88% in 10hr by Py1, 84% in 14hr by Py4, and 88% in 8hr by the consortium. It was also determined that the best degradation temperatures were 37°C and pH 7.0 respectively. The influence of different nutrient substrates added in the degradation experiments was also studied. It was shown that sodium salicylate, sodium acetate and yeast extract had obvious simulative effect, but glucose had no obvious effect.

Keywords polycyclic aromatic hydrocarbons, pyrene, *Bacillus sp.*, biodegradation

1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are kinds of refractory organic pollutants that widely exist in the environment. They normally have more than two phenyl rings. In addition, with increasing of the phenyls, their hydrophobicity and half-life in the environment will also be greatly increased. For instance, the more phenyl rings there are, the more genetically toxicity and carcinogenesis PAHs have, and they have the highest genetic toxicity when there are four or five phenyl rings (Guo et al., 2000). At present, the trend is that PAHs in the environment to quickly increase with exploration and usage of petroleum, coals, and natural gas. Therefore, due to PAHs' potential toxicity, carcinogenesis, and aberrance, they have obvious ecological risk to human health and the environment.

Pyrene is one of the four-ring PAHs that widely exist in the environment and it is a reference substance in PAHs detection. Pyrene has been proven to be one of the carcinogenic PAHs (Gong and Li, 2001). Moreover, its metabolite quinone has much stronger toxicity has been reported to be aberrant (Ravelet et al., 2000).

In the present study, two bacterial strains, Py1 and Py4, were tamed and isolated through long cultivation with pyrene as the single carbon source. The two strains were preliminarily determined based on the morphology of their colonies and physiological and biochemical characteristics. Furthermore, the best temperature and pH value of pyrene biodegradation and the impact of co-metabolites were determined.

2 Materials and methods

2.1 Materials and chemicals

2.1.1 Materials

Mineral synthetic medium [MSM, 1000 mL MSM includes 14 g K_2HPO_4, 6.0 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 5.0 g NaCl, 2.0 g $(NH_4)_2SO_4$], and 1 mL trace element solution [TE Tris-ethylenediamine tetraacetic acid, 1000 mL of TE solution includes 1.0g FeSO4, 1.0g $MnSO_4 \cdot H_2O$, 0.25 g $Na_2MoO_4 \cdot 2H_2O$, 0.1 g H_3BO_4, 0.25 g $CuCl_2 \cdot 2H_2O$, 0.25 g $ZnCl_2$, 0.1 g NH_4VO_3, 0.25 g $Co(NO_3)_2 \cdot 6H_2O$, 0.1 g $NiSO_4 \cdot 6H_2O$, and 5 mL of concentrated H_2SO_4), pH 7.0–7.2]; brewis-peptone medium (BP, 1000 mL of BP medium includes 5.0 g brewis, 10 g peptone, and 10 g NaCl, pH 7.0–7.2); and Brewis-Peptone Agar medium (BPA, addition of 15 g agar in 1000 mL of BP medium), autoclave 20 mins at 121°C and 0.1 MPa.

Translated from *Acta Scientiarum Naturalium Universitatis Nankaiensis* (*Natural Science Edition*) 2006, 39: 71–74 [译自: 南开大学学报 (自然科学版)]

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Phosphate Buffer: 0.02mol·L^{-1} NaH_2PO_4-0.02mol·L^{-1} Na_2HPO_4, pH 7.0, autoclave 20 mins at 121°C and 0.1 MPa.

2.1.2 Chemicals and equipments

Pyrene was dissolved in acetone to make a series of standard solutions, and the working solutions were diluted with the standard solutions.

The chemicals and equipments include 960 MC (microcystins) fluorescent spectrophotometer (Shanghai Precision & Scientific Instrument Co., Ltd), TDL-5 bench-top centrifuge (Shanghai Anting Scientific Instrument Co., Ltd), and THZ-22 bench-top incubator (Jiangsu Taicang Experimental Instrument Company).

2.2 Experimental methods

2.2.1 Screening assay

The bacterial consortium, isolated from the bottom silt of shrimp ponds, was incubated in batches at 37°C with 150 r·min^{-1} of shaking using pyrene as the single carbon source. The concentration of pyrene was gradually increased and fresh medium was changed every two days. The highest tolerant concentration of pyrene is at 200 mg·mL^{-1} and it was obtained when the bacteria stopped growing when the concentration is increased further. We screened out the strains in this concentration, and used selective plates several times to obtain pure strains.

An MSM medium was used for domestication, screening of pyrene degrading strains and pyrene degradation experiments while BP and BPA mediums were used for bacterial isolation and purification.

Preparation of bacterial suspension: a bacterial strain was inoculated in the 50 mL BP medium and incubated at 37°C for 24 hrs with shaking. Aliquots of 5 mL bacterial culture were added in 15 mL sterile centrifuge tubes then centrifuged at 4000 r·min^{-1} for 10 mins. The supernatant was poured out and the cell pellets were washed three times with phosphate buffer. The pellets were centrifuged again and resuspended in phosphate buffer. All the above operations were performed under sterile conditions.

In this study, we used a normal category method (Du, 1992) to identify the genera and genus of the bacterial strains based on the morphological, physiological and biochemical characteristics of the bacteria.

2.2.2 Pyrene measurement assay (Jin et al., 2002)

A pyrene-acetone solution (13 mg·mL^{-1}) was added in the 90 mL sterile MSM medium and inoculated with 10 mL bacterial suspension solution which was pre-incubated to exponential phase. The above culture was incubated at 37°C with 150 r·min^{-1} of shaking. The concentration of pyrene residue was measured by fluorescent spectrophotometer (with the wavelength of 333 nm for excitation and 390 nm for detection) every two hours until no more pyrene was degraded. The characteristics of pyrene degradation by the strains Py1, Py4 and their consortium were measured respectively. This assay can detect 0–130 μg·L^{-1} of pyrene in water solution, in which the pyrene concentration was linear to the intensity of fluorescence (Conc = 0.4911 × I-0.0617, R^2 = 0.99), and the detection limit was 0.5 μg·L^{-1}.

The solubility of pyrene in a water solution is very low, it is only at 135 μg·L^{-1}. Moreover, most of the known pyrene degrading bacteria can only degrade soluble pyrene. Thus, 130 μg·L^{-1} of pyrene was used as the initial concentration in all the biodegradation experiments to obtain a homogenous, soluble pyrene solution to make the operation convenient. Since the cell debris is prone to adsorb pyrene due to their hydrophobicity, the following experiments were performed to determine if the adsorption of pyrene by the cell debris was important: the bacteria culture was incubated overnight under the same condition described above, and then the concentrations of pyrene in the supernatant and cell pellet after extraction by acetone were measured. Results showed that only 0.66% of the cell debris was in the supernatant after the adsorption of pyrene. Consequently, adsorption was not used in the study.

3 Results and discussion

3.1 Identification of highly-effective degradation bacterial strains

Two strains, Py1 and Py4, were isolated by the screening assay mentioned above. It was found that the colonies of Py1 were round, white, and had secretions; they were gram positive and had endospores; the cells were shaped like long rods and the endospores, which were found in the middle of the cell, were not intumescent and with obtuse ends. For the colonies of Py4, it was found that they were round, white, and had secretions; they were gram positive and had endospores; the cells were shaped like short rods and the endospores, which were found in the middle of the cell, were elliptical, not intumescent and with obtuse ends. In addition, both of the strains were found to be aerobic or facultative anaerobic, and chemoheterotrophic bacteria. The physiological and biochemical characteristics of the strains are listed in Table 1. Based on the Gordon Taxonomy, Py1 and Py4 were preliminarily identified as the first group of *Bacillus*: sporangium; they are not obviously intumescent; their endospores, which may be found in the middle or at the end of the cell, are either round or rod-shaped; they are gram positive.

3.2 Degradation characteristics

The strains Py1 and Py4 as well as their consortium of were incubated as described above and the concentrations of pyrene were measured every two hours using fluorescent spectrophotometer. The variation of the pyrene concentration

Table 1 Characteristics of physiology and biochemistry of strain Py1 and Py4

Characteristics	V-P reaction	Hydrogen peroxide ase	Oxidase	Amylolytic	Degradation of tyro protein	Nitrate reduction	β-galacto sidase	Amino acid dec arboxylase	Cytochrome	Urease
Py1	+	+	–	+	+	+	+	+	–	+
Py4	+	–	+	+	+	+	+	+	+	–

was proportional to the variation of the relative fluorescent intensity. Autoclaved medium without bacteria was used as blanked control. Thus, we obtained the degradation curves of Py1, Py4 and their consortium using the relative pyrene residue (%) versus the incubation time (hrs) as described in Fig. 1.

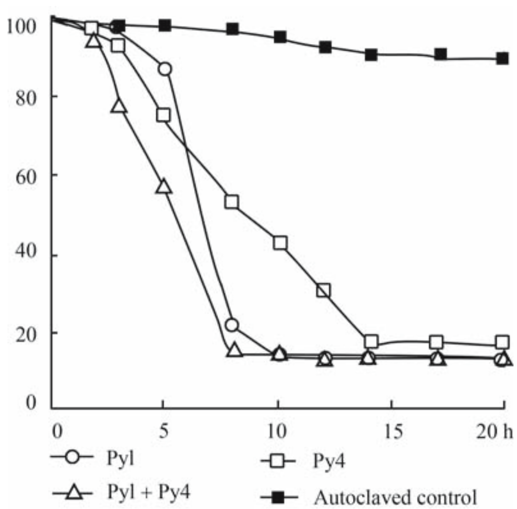

Fig. 1 Biodegradation curves of pyrene by Py1, Py2 and the consortium

Figure 1 showed that for 8 hours incubation the pyrene degradation rate by Py1 and Py4 were 78.5% and 48%, respectively, while the degradation rate by their consortium was as high as 86%. After inoculation, the lag phase of Py1 was longer than Py4, the degradation rate in exponential phase was much higher and final degradation rate was as high as 88%,. While the speed of the pyrene degradation of Py4 was much lower than that of Py1, its final degradation rate was still 84%. Moreover, the consortium showed obvious advantage than the single strain. They had shorter lag phase and higher degradation speed. Its final degradation rate was also 88%.

It is really difficult to biodegrade pyrene in nature. Usually, the degradation rate of pyrene by wild type bacteria in the environment is very low when without additional carbon and energy sources.

For example, Gong and Li (2001) only achieved 57% of pyrene degradation rate in their study; Ramirez et al. (2001) reported their pyrene degradation rate as 63%; and Zhang et al. (2002) achieved a high pyrene degradation rate of 75.6%. All results are still lower than the results we have achieved in our study. Therefore, the two strains should be considered as the highly-effective pyrene degradation bacteria.

3.3 Optimization of biodegradation temperature and pH value

To obtain the optimized degradation condition in our experimental system, the pyrene degradation rates were measured at different incubation temperatures of 20°C, 30°C, 37°C, and 40°C, and at different pH values of 5.0, 6.0, 7.0, 8.0, and 9.0. Due to the obvious advantage of the consortium over the single strain, only the consortium was used to optimize the incubation temperatures and pH values (Figs. 2 and 3). Figures 2 and 3 showed that 37°C and 7.0 were the best degradation temperature and pH value respectively.

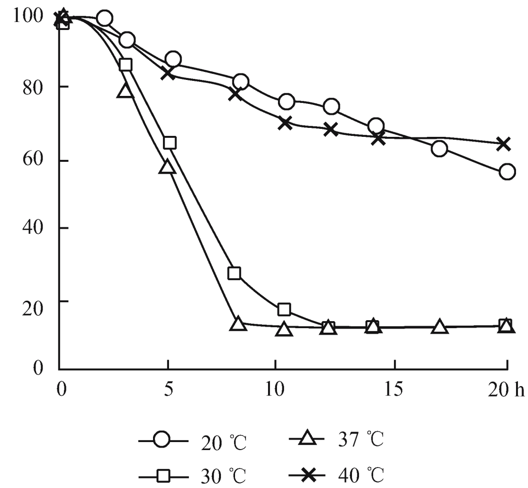

Fig. 2 Effects of temperature on degradation rate

3.4 Functions of the co-metabolites

In the process of PAHs degradation, the oxidation and the breaking of the phenyl rings are always the initial reactions in biodegradation. Although these cause the bottlenecking of PAHs biodegradation by microorganisms, the progress is strictly forward thereafter and there are almost no accumulation of intermediate metabolites. It was found that a premix with some co-metabolites would increase some specific enzyme activities and greatly expedite the biodegradation process of pyrene. It was also found that the reaction rate was relatively higher in the initial stage and that the reaction rate would be decreased with the progressing reactions, in which the reaction will be accorded with first order dynamic mode

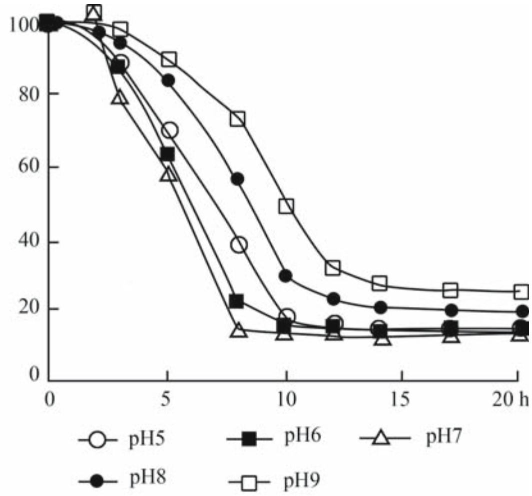

Fig. 3 Effects of pH value on degradation rate

(Gong and Li, 2001; Ramirez et al., 2001; Ting et al., 2001; Yuan et al., 2000).

50 mg · L^{-1} of co-metabolites (glucose, sodium salicylate, sodium acetate, and yeast extract) was premixed with the medium described above, and was then used to in biodegradation experiments. The degradation results in Fig. 4 showed that the sodium salicylate, sodium acetate, and yeast extract facilitated pyrene biodegradation, while glucose did not show any significant effect. Hence, the trend of co-metabolite to facilitate biodegradation could be described as:

yeast extract > sodium salicylate > sodium acetate > glucose

where the addition of yeast extract achieved the highest pyrene biodegradation rate.

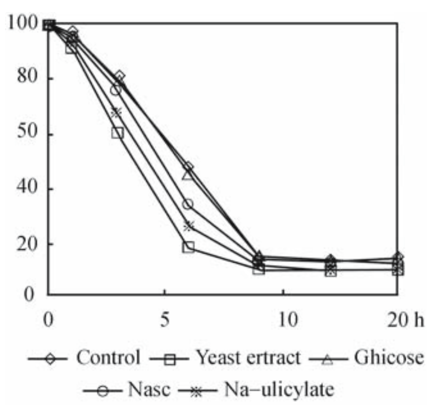

Fig. 4 The degradation results of pyrene by the consortium under existence of CO-substrates

4 Conclusions

In this study, the two isolated *Bacillus* strains can utilize pyrene as a single carbon source, can effectively and quickly degrade soluble pyrene. Moreover, their consortium (1:1) can achieve higher degradation speed than any single strain. The possible reason is that the consortium shared their enzyme systems to supply the required conditions for their growth and eliminate the obstacles in their metabolism hence expediting pyrene biodegradation.

The optimized degradation rate that was achieved at 37°C and pH 7.0 was determined by comparing the degradation characteristics under different temperatures and different pH values using the consortium. Furthermore, when four co-metabolites (glucose, sodium salicylate, sodium acetate, and yeast extract) were added to the experimental system, it was found that they had different effects on pyrene biodegradation. While sodium salicylate, sodium acetate, and yeast extract obviously expedited the degradation and the addition of yeast extract achieved the highest pyrene degradation rate, glucose did not show any significant effect.

In this study, it was found that pyrene could not be totally degraded and that there was always 3.0 μg · L^{-1} of pyrene residue in the solution. Even with the addition of co-metabolites and optimized conditions, there was still 2.25 μg · L^{-1} of pyrene residue left. This low concentration is called the threshold. We hypothesize that this concentration of pyrene is extremely low and cannot be utilized by microorganisms as a carbon source. We therefore conclude that the further reduction of the residue threshold and minimization of the ecological risk of PAHs like pyrene have become crucial issues in environmental science and that they have practical implications for human health and the environment.

Acknowledgements This paper was supported by the National Natural Science Foundation of China (Grant No. 402173036).

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