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Full Length Research Paper

Biodegradation of phenanthrene in artificial seawater by using free and immobilized strain of *Sphingomonas* sp. GY2B

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Biodegradation has been suggested as an alternative way to remove polycyclic aromatic hydrocarbons (PAHs) from contaminated environment. Phenanthrene is a representative carcinogenic PAHs containing "bay-region" and "K-region". Strain *Sphingomonas* sp. GY2B is a high efficient phenanthrene-degrading strain isolated from crude oil contaminated soils and had a broad-spectrum degradation ability on PAHs and related aromatic compounds. This paper reports the domestication of strain *Sphingomonas* sp. GY2B in artificial seawater (AS) and the immobilization of the strain onto rice straw. Results showed that adding 85% artificial seawater had very low impact on the growth and phenanthrene degradation ability of strain GY2B being domesticated for five generations. Phenanthrene was rapidly degraded when the growth of strain GY2B was in the exponential phase that the initial added 100 mgL^{-1} phenanthrene had been almost completely degraded within 66 h. The optimal immobilization carrier weight and length of rice straw were 25 gL^{-1} and 0.5 cm, respectively. The immobilized strain GY2B had high degradation rate both in mineral salts medium and 80% artificial seawater, and was higher than that of the free strain GY2B. More than 95% phenanthrene (100 mgL^{-1}) was degraded within 32 h, and the phenanthrene degradation percentages were > 99.5% after 67 h for immobilized strains. Immobilization of strain GY2B with rice straw possesses a good application potential in the treatment of wastewater and bioremediation of estuary and offshore environment contaminated by phenanthrene.

Key words: Artificial seawater, biodegradation, immobilization, phenanthrene, polycyclic aromatic hydrocarbons, rice straw, *Sphingomonas* sp. GY2B.

INTRODUCTION

Phenanthrene is now one of the most common environmental pollutants, which is a component of fossil fuels and can be formed from incomplete combustion of organic matter (Juhasz and Naidu, 2000). Phenanthrene is hazardous to aquatic life, plants and many other organisms and has often been used as a representative carcinogenic polycyclic aromatic hydrocarbons (PAHs) containing "bay-region" and "K-region" (Samanta et al., 1999). Thus, degradation of phenanthrene effectively is necessary to preserve the environment and the health of human beings.

Efficient treatment methods are available for the degradation of phenanthrene (Cataldo and Keheyan, 2006; Little et al., 2002; Lu et al., 2007; Samanta et al., 2002; Sirisaksoontorn et al., 2009). Comparing with physico-chemical methods, biodegradation is regarded as an alternative method to detoxify or remove phenanthrene

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Abbreviations: **PAHs**, polycyclic aromatic hydrocarbons; **MSM**, mineral salts medium; **AS**, artificial seawater; **PDR**, phenanthrene degradation rate.

from the environment because of lower costs and the possibility of complete mineralization (Cerniglia, 1992). In this sense, biodegradation of phenanthrene has therefore gained an increasing attention and a large number of phenanthrene-degrading bacteria such as *Sphingomonas* (Tao et al., 2007a; Zhao et al., 2009), *Pseudomonas* (Prabhu and Phale, 2003; Tian et al., 2002), *Mycobacterium* (Miller et al., 2004; Stingley et al., 2004), *Nocardioides* (Iwabuchi et al., 1998; Saito et al., 2000) and *Sinorhizobium* (Keum et al., 2006) species, have been isolated and characterized at the physiological and genetic level.

Sphingomonas sp. was shown to have unique genes for degradation of phenanthrene and other PAHs (Pinyakong et al., 2003a). The gene is distantly related to those of *Pseudomonas* and other genera reported so far in sequence homology and genorganization (Pinyakong et al., 2003b). Strain *Sphingomonas* sp. GY2B was shown to efficiently use phenanthrene as the sole carbon and energy source that above 99.1% of phenanthrene at initial concentration of 100 mgL^{-1}, phenanthrene could be degraded at 30°C within 2 days. In addition to phenanthrene, strain GY2B could use a broad range of PAHs and related aromatic compounds such as naphthalene, 2-naphthol, salicylic acid, catechol, phenol, benzene and toluene as a sole source of carbon and energy (Tao et al., 2007b).

The use of free bacterial cells for bioremediation of contaminated sites might fail since the inoculants must be able to overcome biotic and abiotic stresses in the environment in which they are introduced, and might cause other problems such as secondary pollution by the inoculants (Gentili et al., 2006). Immobilized microorganisms have been shown to be effective and have been receiving increasing attention (Liu et al., 2009; Tao et al., 2009; Wang et al., 2008; Zhang et al., 2008). The purpose of this work is to study the biodegradation of phenanthrene in artificial seawater (AS) by using free and immobilized cells of *Sphingomonas* sp. GY2B, and to explore the environmental adaptability and potential application of the strain.

MATERIALS AND METHODS

Materials and growth conditions

Phenanthrene-degrading strain *Sphingomonas* sp. GY2B was isolated from crude oil contaminated soils collected at a site near Guangzhou Petrifaction Company, China (Tao et al., 2007a). Rice straw was used as immobilization carrier and was obtained from local farmers in Guangzhou, China. Phenanthrene was purchased from Aldrich Company (USA, purity above 98%) and its hexane stock solution at 10 gl^{-1} was prepared and stored in brown bottle placed at 4°C. All other chemicals used were of the highest purity available. The phenanthrene-degrading strain was routinely grown at 30°C in mineral salts medium (MSM) consisting of the following (per liter): 5 mL phosphate buffer solution (KH_2PO_4, 8.5 gL^{-1}; $K_2HPO_4 \cdot H_2O$, 21.75 gL^{-1}; $Na_2HPO_4 \cdot 12H_2O$, 33.4 gL^{-1}; $(NH_4)Cl$, 5.0 gL^{-1}); 3.0 mL $MgSO_4$ solution (22.5 gL^{-1}); 1.0 mL $FeCl_3$ solution (0.25 gL^{-1}); 1.0 mL $CaCl_2$ solution (36.4 gL^{-1}); 1.0 mL trace element solution ($MnSO_4 \cdot H_2O$, 39.9 mgL^{-1}; $ZnSO_4 \cdot H_2O$, 42.8 mgL^{-1}; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 34.7 mgL^{-1}). Its pH was adjusted to 7.2 - 7.4 with 5 mol L^{-1} HCl and NaOH solutions. AS was used for the domestication of phenanthrene-degrading strain. The AS consisted of the following (per liter) (Lyman and Fleming, 1940): 24.5 g NaCl; 0.03 g H_3BO_3; 1.54 g $CaCl_2 \cdot 2H_2O$; 4.09 g Na_2SO_4; 0.1 g KBr; 0.003 g NaF; 0.2 g $NaHCO_3$; 0.7 g KCl; 0.017 g $SrCl_2 \cdot 6H_2O$; 11.1 g $MgCl_2 \cdot 6H_2O$. Its pH was adjusted to 7.5 with 5 mol L^{-1} HCl and NaOH solutions. Nutrient agar consisted of 10 gL^{-1} peptone, 5 gL^{-1} beef extract, 5 gL^{-1} NaCl and 2.0% agar. Estimation of the cell growth was made according to colony-forming units (CFU) plate-counting on nutrient agar. All the plates with microorganisms were placed at 30°C.

All the media and solutions were prepared with distilled water and autoclaved at 1 atm for 15 min. Strain GY2B was activated in MSM with 100 mgL^{-1} phenanthrene for 2 days before each immobilization and degradation experiments. All experiments were carried out in 100 mgL^{-1} phenanthrene except those especially illuminated.

Domestication of the strain in AS

Growth of the strain in increasing concentration of AS was investigated to assess its salinity adaptability. First, 0.5 mL hexane stock solution of phenanthrene was added to sterilized flasks, forming a thin film of phenanthrene on the flask bottom after evaporation of hexane, then sterilized MSM and AS (50:50, v/v) was added. The flasks with 45 mL mixed medium of 50% AS were inoculated with 5 mL cell culture induced by phenanthrene and then were incubated at 30°C on a shake preset at 150 rpm under dark condition. After 3 days, both phenanthrene degradation and cell growth were tested, and simultaneously, an aliquot of 5 ml supernatant was transferred to another flask with mixed medium consisting of higher proportion of AS for a second domestication. This domestication procedure was repeated five times and the AS proportion was added up to 85%. Flasks having no AS, had no bacterial strain were set up as control reactors. All assays were carried out in duplicate.

Immobilization of the strain with rice straw

Before any pre-treatment, rice straw was washed thoroughly with tap water until the washings were clean and colourless and then air dried for further treatment. Air-dried rice straw was cut to 1.0 and 0.5 cm in length, respectively, and some was also ground into powder. Then 1.5, 2.5 and 3.5 g dry weight sterilized rice straw were added into flasks containing 90 ml MSM + 10 ml activated strain GY2B culture with 100 mgL^{-1} phenanthrene as sole carbon source, respectively. The flasks were incubated at 30°C on a shaker preset at 150 rpm under dark condition for 36 h and then the residual phenanthrene was extracted and determined to decide the optimal adding length and weight of the carrier.

Biodegradation tests with free and immobilized strains

The immobilized strain GY2B that grew in the optimal rice straw addition for 2 days at the late exponential phase, were harvested with gauze and rinsed with normal saline (0.90%) twice. The biodegradation of phenanthrene in 100 ml (including inoculant culture) 80% AS was investigated by inoculating 10 ml activated cell culture, 2.5 g of 0.5 cm rice straw, 10 ml activated cell culture and 2.5 g of 0.5 cm rice straw and preimmobilized strain in 2.5 g of 0.5 cm rice straw, respectively. The flasks were incubated at 30°C on a shaker preset at 150 rpm under dark condition for 54 h and then the residual phenanthrene was extracted and detected. In addition, the harvested carriers of equal amount were inoculated to the flasks

containing sterilized MSM or 80% AS media with 100 mgL^{-1} phenanthrene as sole carbon source, and incubated at 30℃ and 150 rpm. Flask samples were taken at 0, 8, 22, 32, 44, 52, 67 and 72 h to detect the residual phenanthrene in the flask. Flasks having no inoculant were set up as control test. All assays were carried out in triplicate.

Analysis methods

Quantitative analysis of phenanthrene in the flask was performed by extracting the whole flask cultures with 20 ml cyclohexane twice. The two extracts were combined and dried with anhydrous Na_2SO_4. The solvents were diluted with cyclohexane to certain volumes (or after evaporated under reduced pressure). The detection of phenanthrene in cyclohexane solution was performed using UV-Vis spectroscopy (UV752N) by measuring the absorbance at 254nm (OD_{254}) (Little et al., 2002). The linearity range of this method in 0.04 - 4.0 mgL^{-1} is quite good because the correlation coefficient between the phenanthrene concentration and OD_{254} was always > 0.999.

Control tests with varied phenanthrene concentrations were run to assess the recovery efficiency under extraction condition, which was > 85% at phenanthrene concentration of 0.01 mgL^{-1}. One-way ANOVA test was employed to estimate the significant differences of different assays at the 0.05 level by using statistical analysis software OriginPro 7.0.

RESULTS

Domestication in AS

Through stepwise increasing of AS proportion, the five generation of strain GY2B could grow well in 85% AS and efficiently degrade phenanthrene at initial concentration of 100 mgL^{-1}. The growth of strain GY2B and phenanthrene degradation curves are shown in Figures 1 and 2, respectively.

Comparing with pure MSM incubation of initial strain GY2B, adding 85% AS had very low impact on the growth and phenanthrene degradation ability of domesticated strain GY2B. As can be seen in Figure 1, both growth trends in these two conditions were almost the same. Live cells had increased about 20 times within 66 h incubation and then the growth of the strain went into decline phase. Figure 2 shows that phenanthrene was rapidly degraded when the growth of GY2B was in the exponential phase that the added phenanthrene had been almost completely degraded within 66h. On the exponential phase of GY2B, more than 60% phenanthrene was degraded within 18 h and the phenanthrene degradation percentages were close to 98% after 42 h.

Optimal immobilization condition

To determine the optimal carrier length and additional weight, different immobilization conditions were investigated. The residual phenanthrene after 36 h incubation in different immobilization length and weight of carrier is as shown in Figure 3. Results showed that the

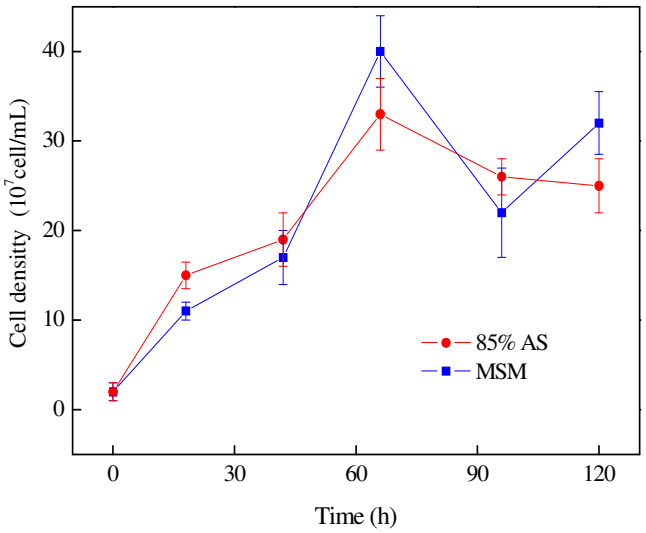

Figure 1. Effect of artificial seawater on the growth of free strain GY2B.

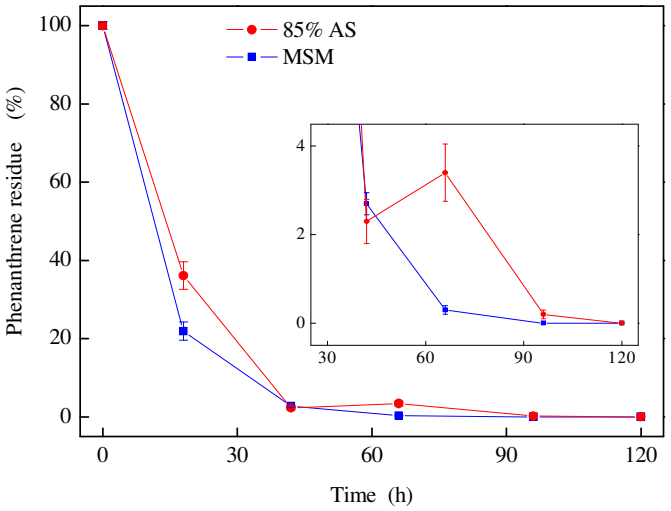

Figure 2. Effect of artificial seawater on the phenanthrene degradation of free strain GY2B.

phenanthrene degradation percentages were > 98% for all tests. The 0.5 cm rice straw addition tended lowest phenanthrene residue in each carrier length experiment and the lowest phenanthrene residue (1.00 mgL^{-1}) appeared in the 25 gL^{-1} dry weight 0.5 cm rice straw experiment. Therefore, 25 gL^{-1} of 0.5 cm rice straw was selected as the optimal immobilization condition for further experiments.

Comparison of free and immobilized strains

The residual phenanthrene after 54 h incubation in

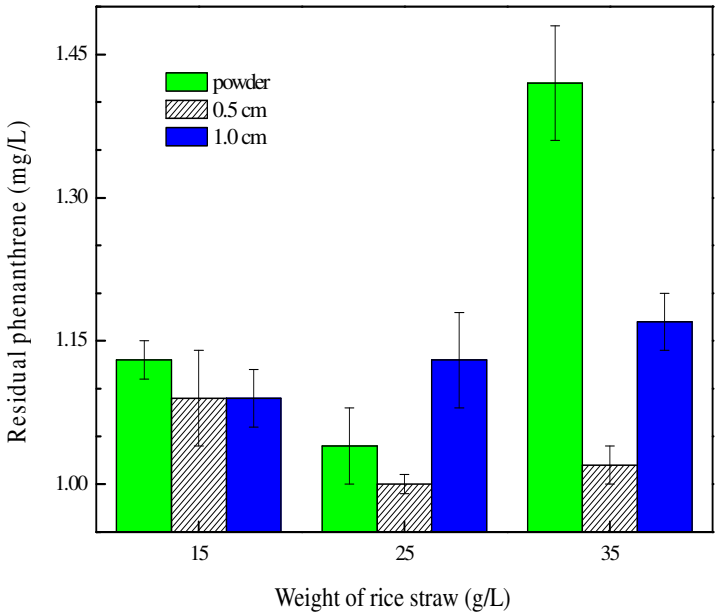

Figure 3. Effect of carrier length and weight on the degradation of phenanthrene.

Table 1. Phenanthrene degradation percentages of free and immobilized strain GY2B in 80% AS.

Inoculants	% Phenanthrene degradation (54h)
Rice straw (2.5 g, 0.5 cm) with no strain	12.67 ± 0.31 a
Free GY2B cell culture (10 ml)	98.58 ± 0.14 b
Rice straw (2.5 g, 0.5 cm) + Free GY2B cell culture (10 ml)	98.65 ± 0.17 bc
Preimmobilized GY2B in rice straw (2.5 g, 0.5 cm)	98.95 ± 0.10 c

Means ± SD followed by the same letter within the column are not significantly different at the 0.05 level according to one-way ANOVA test.

different inoculants is shown in Table 1. Results showed that both free suspended culture and immobilized strain had high phenanthrene degradation efficiencies in 80% AS, higher than 98% within 54 h with an initial concentration of 100 mgL^{-1} phenanthrene. Comparing with free strain, immobilized strain exhibited a slight larger phenanthrene degradation percentage, which indicated that the immobilization of strain GY2B can improve its phenanthrene degradation efficiency.

Biodegradation test of immobilized strain

To confirm that immobilized strain can also tolerate a wide salinity changes, phenanthrene degradation experiments were performed in MSM and 80% AS media with 100 mgL^{-1} phenanthrene as sole carbon source, respectively. The phenanthrene degradation trends of free and immobilized strain GY2B in MSM and 80% AS media are shown in Figure 4. Results showed the immobilized strain has large degradation percentage in both media, and the immobilized strain has larger phenanthrene degradation percentage than the free strain in 80% AS. More than 95% phenanthrene was degraded within 32 h, and the phenanthrene degradation percentages were > 99.5% after 67 h for immobilized strains. The phenanthrene degradation percentages of immobilized strain was larger than that of the free strain through the whole process. These results indicate that immobilized *Sphingomonas* sp. GY2B possesses a good application potential in the treatment and remediation of phenanthrene-contaminated wastewater and seawater.

DISCUSSION

For better comparison of the biodegradation efficiency by immobilized strain GY2B with the free strain, the degradation data were further reduced to obtain the phenanthrene degradation rate (PDR) during the linear-decreasing stage of incubation with the following equation and listed in Table 2.

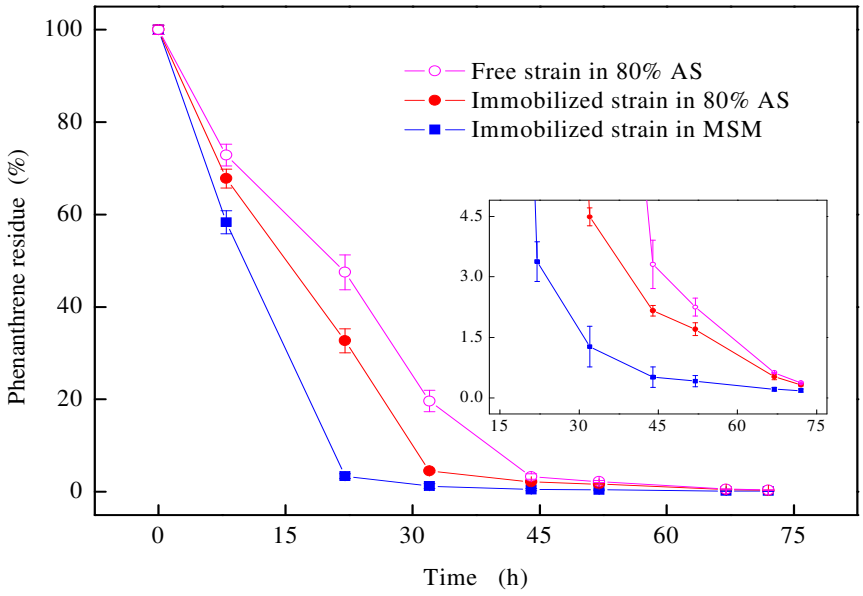

Figure 4. Phenanthrene degradation trends of free and immobilized strain GY2B.

Table 2. Phenanthrene degradation rates (PDR) of free and immobilized strain GY2B [1].

Inoculants [2]	Media	Incubation period (h) [3]	$c(0)$ (mgL^{-1})	$c(t)$ (mgL^{-1})	PDR (mgL^{-1}h^{-1})
Free▲	MSM	0~42	100 a	2.30 ± 0.50 a	2.33 ± 0.04 a
Free▲	85% AS	0~42	100 a	2.70 ± 0.25 ab	2.32 ± 0.03 a
FreeΔ	80% AS	0~22	100 a	47.5 ± 3.78 c.	2.38 ± 0.15 a
ImmobilizedΔ	80% AS	0~22	100 a	32.7 ± 2.61 d	3.06 ± 0.11 b
ImmobilizedΔ	MSM	0~22	100 a	3.38 ± 0.49 b	4.39 ± 0.07 c

[1]Means ± SD followed by the same letter within each column are not significantly different at the 0.05 level according to one-way ANOVA test.
[2]Labels ▲ and Δ represent two different experimental batches; [3]the linear-decreasing time interval involved in the calculation of PDR.

$$\text{PDR} = \frac{c(0) - c(t)}{t} \quad (1)$$

Where c presents the concentration of phenanthrene and t is the rapid linear-decreasing degradation period. The calculated results showed the PDR of free strain GY2B is about 2.30 mgL^{-1}h^{-1} and the PDR of immobilized strain GY2B is 3.06 and 4.39 mgL^{-1}h^{-1} in 80% AS and MSM, respectively. These results clearly showed the negative effect of AS and the enhancement of immobilization on the phenanthrene degradation efficiency of strain GY2B. Compared with the free strain GY2B in 80% AS, the immobilization has enhanced by > 28% PDR.

Marine culture is thriving in some countries and represents a major component of the regional economy in coastal zones, yet the environmental quality of many of these areas is poor. Zhou and Maskaoui (2003) investigated the PAHs in surface water and sediments of Daya Bay, China and found that the total concentrations of 16 PAHs varied from 4228 to 29325 ngL^{-1} in surface water (153 - 1445 ngL^{-1} phenanthrene) and from 115 to 1134 ng g^{-1} dry weight in sediments (1 - 94 ngL^{-1} phenanthrene). The PAH levels at six sites of Daya Bay waters were sufficiently high (> 10 μgL^{-1}) to cause acute toxicity. Similar high levels were also found in some seawater samples around England (Law et al., 1997). Although *Sphingomonas* sp. GY2B was isolated from terrestrial soils (Tao et al., 2007a), this study showed it could grow well and keep high effective phenanthrene degradability in relatively high salinity condition. Salinity is one of the most important abiotic factors in aquaculture (Ye et al., 2009). The AS salinity used in this study is about 37%, so the salinity of 85% AS should be > 31%, larger than the salinity in most estuary and offshore surface waters (Bjorlykke and Gran, 1994; Yin et al., 2001; Zhou and Maskaoui, 2003). Hence, strain GY2B has good application potential in bioremediation of estuary and offshore environment polluted by PAHs.

Using rice straw as immobilization carrier is economically cheap and environmentally friendly for potential application of strain GY2B (Nguyen et al., 1994; Abdel-Mohdy et al., 2009). The immobilized strain GY2B on raw rice straw has higher phenanthrene degradation efficiency than the free strain in this study, which might result from the adsorption of phenanthrene onto the carrier promoting the degradation efficiency of the strain immobilized on it. Studies showed that modified rice straw is a good biosorbent for some pollutants in water, such as oil-spills (Sun et al., 2002), dyes (Gong et al., 2008) and heavy metal ions (Rocha et al., 2009). Modification of rice straw might enhance the adsorption of phenanthrene onto the carrier and further improve the phenanthrene degradation efficiency of strain GY2B; further investigation on this issue is in progress.

Conclusion

Domestication of phenanthrene-degrading strain *Sphingomonas* sp. GY2B in AS was carried out and the phenanthrene degradation efficiencies of free and immobilized strain GY2B were investigated. Results showed that adding 85% artificial seawater had very low impact on the growth and phenanthrene degradation ability of domesticated strain GY2B. The optimal immobilization carrier weight and length of rice straw were 25 gL^{-1} and 0.5 cm, respectively. The immobilized strain GY2B had high degradation rates both in MSM and 80% AS, and was higher than that of free strain GY2B. Immobilization of strain GY2B with rice straw possesses a good application potential in the treatment of wastewater and bioremediation of estuary and offshore environment contaminated by phenanthrene.

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