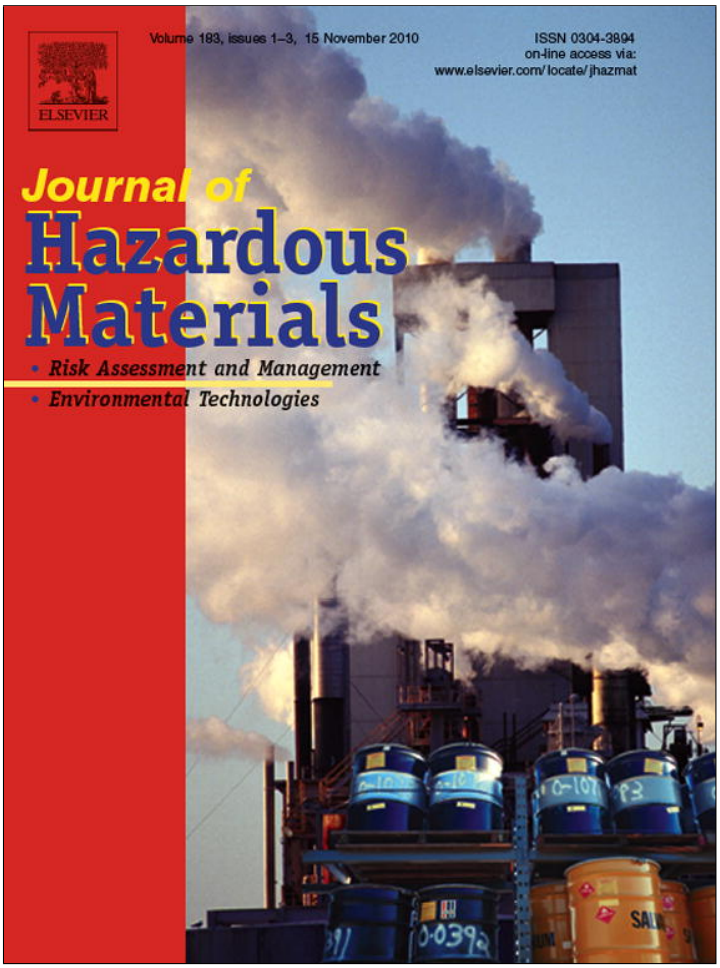

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Promoted biodegradation and microbiological effects of petroleum hydrocarbons by *Impatiens balsamina* L. with strong endurance

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ABSTRACT

Phytoremediation is a promising green technology for cleanup of petroleum hydrocarbons (PHCs) in contaminated environment. Based on the objective of identifying special ornamental plants for the effective biodegradation of PHCs, the efficacy of *Impatiens balsamina* L. to phytoremedy petroleum contaminated soil from the Shengli Oil Field in Dongying City, Shandong Province, China, was further examined in a field plot-culture experiment under greenhouse conditions. After a 4-month culture period, the average degradation rate of total petroleum hydrocarbons (TPHs) by the plant was up to 18.13–65.03%, greatly higher than that (only 10.20–35.61%) in their corresponding controls by natural degradation. Among petroleum compositions saturated hydrocarbons had the highest degradation. The release of polar metabolic byproducts during phytoremediation of contaminated soils with ≥20,000 mg/kg of PHCs by *I. balsamina* may occur. Some growth indexes of *I. balsamina* indicated that the plant had a good tolerance to contaminated soils with ≤10,000 mg/kg of PHCs. Moreover rhizosphere bacteria and fungi became the dominant microbial population in soils with 5000 and 10,000 mg/kg of PHCs and were probably responsible for TPH degradation. Thus, *I. balsamina* L. could be a potential ornamental plant for effective phytoremediation of contaminated soils with ≤10,000 mg/kg of PHCs.

1. Introduction

Petroleum hydrocarbons (PHCs) are an important category of environmental contaminants in industrialized countries. Although petroleum supplies the largest share of world energy consumption, during the processes of exploration, extraction, refining, transporting and marketing petroleum products, an increasing area of lands has been seriously polluted by PHCs in the world [1,2]. Accumulation of PHCs in the environments is now threatening human health and ecosystem safety, including inhibiting plant growth, destroying soil structures, and damaging groundwater quality [3]. Therefore, remediation of petroleum hydrocarbon contaminated soils is becoming one of the hot topics in the field of environmental science and engineering [4,5].

Phytoremediation is defined as a promising green technology for removal of pollutants from contaminated environment. It uses plants to degrade, transform, assimilate, metabolize, or detoxify hazardous pollutants from soils, aquatic and atmospheric environments [6]. In comparison with conventional ex situ treating methods, such as excavation and incineration, off-site storage and soil washing, phytoremediation has lots of technical advantages and ecological benefits [4]. In particular, it is inexpensive, in situ, simple and easy to operate, seems to be effective, and cannot result in secondary contamination. Furthermore nutrients and oxygen are also added into soils through plant growth and microbial metabolic processes, which can improve the overall quality and textures of soils during phytoremediation [7,8].

The effectiveness of phytoremediation has been demonstrated in a wide range of applications, especially in phytoremedying hazardous heavy metal-contaminated soils. Until now, over 400 species of terrestrial plants had been identified as hyperaccumulators for heavy metals in the world [9,10]. In recent years, based on its considerable potential there is an increasing interest in broadening application of phytoremediation to remove/degrade petroleum contaminants in the environment [11,12]. The main mechanism of phytoremedying petroleum contaminated soils is assumed to be based on a stimulation of degrading microorganisms in the rhizosphere, called rhizodegradation [13]. It is well documented that rhizodegradation is responsible for the enhanced removal of TPHs from soils by deeply rooted trees and other annual species [14]. Pre-

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vious studies have documented that PHC pollution at high levels has negative effects on plant growth [15], but the low level of PHCs does not inhibit plant growth [16]. Both the phytotoxicity and hydrophobic nature of PHCs prevent their bioavailability and extractability. In the rhizosphere, however, microorganisms could offer external protection to the plant, which in return benefit from root exudates and oxygen put in by roots. This is where plant–microorganism association may play an important role in making PHCs more available for uptake and biodegradation by plants [5,17]. Accumulation of PHCs was found in poplar, pine and grass roots by Palmroth et al. [12]. Radwan et al. [18] also evidenced that long-chain PHCs accumulated in *Vicia faba* L. Phytoaccumulation of PHCs in plants, especially in crops, was thought to pose a potential risk to food chain.

Up to now plants identified and applied to phytoremediation of petroleum contaminated soils have been processed in a number of field and greenhouse studies [5,14]. However, less is reported that ornamental plants have been assessed to phytoremediation of petroleum contaminated soils. Ornamental plants can not only further beautify our environment, but remedy petroleum polluted soils as well. Furthermore, ornamental plants can avoid introducing the contaminant or its metabolites into the food chain. Thereby screening out remediation plants from abundant ornamentals has a practical significance in efficient biodegradation and removal of PHCs in contaminated sites [13,19,20]. According to an elementary screen from ornamental plants, Wang [21] found that *Impatiens balsamina* L. had a high tolerance and accumulation ability to cadmium (Cd) and lead (Pb), showing a great potential for phytoremediation under favorable conditions. Therefore, the objective of this study was to assess the potential of *Impatiens balsamina* L., a special ornamental plant, with its rhizosphere microorganisms for bioremediation of petroleum contaminated soil by analyzing the quantity and quality of residual TPHs, especially with the extremely high concentration (40,000 mg/kg) of PHCs in soils. In the meantime, *I. balsamina* biomass and rhizosphere microbial characteristics in relation to the degradation of TPHs were also evaluated. To our knowledge, this might be the first study on the promoted biodegradation and microbiological effects of petroleum hydrocarbons by *I. balsamina* L. under greenhouse conditions.

2. Materials and methods

2.1. Experimental site and soil preparation

The experimental site was installed in the greenhouse (latitude 39°6′N, longitude 117°9′E) of Agro-Environmental Protection Institute of Ministry of Agriculture, Tianjin, China, which is a temperature zone with a monsoon-influenced humid continental climate. The experiment was carried out inside the greenhouse with approximately 16 h natural daylight. The daily average temperature inside the greenhouse stayed about 18–25 °C.

Weathered petroleum contaminated soils containing about 10% (W_{oil}/W_{soil}) concentration of PHCs in the soils were collected from the Shengli Oil Field in Dongying City, Shandong Province, China. Fractions involved in petroleum hydrocarbons from contaminated soils included 74.18% of saturated fraction, 14.31% of aromatics, and 11.51% of asphaltene and polar fraction. Clean surface (0–20 cm) soil samples from its vicinity passed through a 2-mm sieve, were used in the plot-culture experiment. The chemical and physical properties of the clean soils are listed in Table 1. Air-dried contaminated soils were also passed through a 2-mm sieve and then thoroughly artificially mixed with the different amount of clean surface soil samples in order to obtain the diluted contaminated soils. After the dilution, there were five concentration gradient treatments including 0 mg/kg (0.0%, W_{oil}/W_{soil}), 5000 mg/kg (0.5%, W_{oil}/W_{soil}), 10,000 mg/kg (1.0%, W_{oil}/W_{soil}), 20,000 mg/kg (2.0%, W_{oil}/W_{soil}), and 40,000 mg/kg (4.0%, W_{oil}/W_{soil}), respectively. The composite soils were covered (to reduce PHC volatilization) and equilibrated completely at a low temperature (reduce microbial activity) for about 1 week. After that, the value of composite soil pH was in the range from 7.6 to 7.8 (no significant difference). And the value of organic matter was 14.2, 25.8, 38.4, 58.6, 108.2 g/kg in the composite soil samples, respectively.

2.2. Remediation plant species and plot-culture experiment

Seeds of *I. balsamina* with sealed package were purchased from the Liaoning Institute of Flowers, Shenyang, China. *I. balsamina*, cultivated as an ornamental through the world, is a species of Impatiens probably native to China, India and Myanmar in southern Asia. It is an annual or perennial ornamental with a thick but soft stem. The plant prefers acid, neutral and alkaline soils, requires warm and moist conditions [22] and succeeds in the sun or semi-shade.

The plot was divided into five subplots (length = 100 cm, width = 35 cm, depth = 20 cm). These five subplots were filled with 0, 5000, 10,000, 20,000, and 40,000 mg/kg of petroleum contaminated composite soil samples, respectively in order to form the concentration gradient. Then each subplot was randomly subdivided into four treatment cells (length = 25 cm, width = 35 cm, depth = 20 cm). To minimize the experimental errors, establishment of *I. balsamina* was replicated in three treatment cells within each of five subplots including 0, 5000, 10,000, 20,000, and 40,000 mg/kg of petroleum contaminated composite soil samples, respectively. The corresponding unplanted control was also established in one treatment cell within each of five corresponding subplots including 0, 5000, 10,000, 20,000, and 40,000 mg/kg of petroleum contaminated composite soil samples, respectively.

I. balsamina was pre-cultured in uncontaminated soils in order to minimize phytotoxic effects of PHCs at the first stage of plant growth [23]. After that, seedlings of *I. balsamina* with 10 days old and the similar biomass were established in corresponding cells as designed in the experiment. The distance between each seedling of *I. balsamina* was about 5.0 cm. There were approximately 24 seedlings in each cell. Altogether there were 360 seedlings used in the study. Some seedlings were wounded when they were watered and then removed. However, more than 90% of seedlings survived throughout the experiment. Loss of water by evaporation from plots was made up daily using tap water (no PHCs detected) to keep 60% of the water holding capacity (WHC). Rainfall was not used as a water source for *I. balsamina*. The plants were harvested at the seed-maturation stage. The whole experiment lasted for 4 months. At the end of our experiment, the moisture level of soils was in the range from 18.4% to 22.5%. After the maturation of *I. balsamina*, shoots and roots were separated and washed completely with tap water and rinsed with deionized water, dried with filter paper, weighed their fresh weights. Total plant dry weights were determined upon

Table 1
Chemical and physical characteristics of the clean soils used in the experiment.

Property	Value
pH	7.6
Organic matter (g/kg)	14.20
Total N (mg/kg)	2750
Total P (mg/kg)	110
Cd (mg/kg)	ND[a]
Ni (mg/kg)	6.5
Cu (mg/kg)	16.5
Pb (mg/kg)	ND[a]
Cr (mg/kg)	12

[a] ND, not determined.

reaching constant the weight after drying at 60 °C. The composition soil samples were collected and analyzed for petroleum contaminants after 4 months of plant growth. Then rhizosphere soil samples were collected for counting microorganisms in the root zones.

2.3. Determination of total petroleum hydrocarbons (TPHs) and various fractions

Initial and final total petroleum hydrocarbons (TPHs) were determined based on the US EPA 3550c method [24] and the US EPA 1664 method [25]. At first, 50 g soil samples collected from each treatment cell were homogenised, differently labeled and stored at 4 °C until further analysis. The time for storage was no longer than 10 days and had no effect on the determination of TPHs. The soil samples were air-dried at room temperature in the dark and sieved through a 100 mesh. Then 5 g soil samples taken from the 50 g soil samples were analysed further. About 20 ml of dichloromethane (boiling point: 40 °C) was added to 5 g of soil samples, as the extraction solvent in the 40 ml glass tube. After having agitated by a glass stirring rod for 1 min the dichloromethane/soil suspension was extracted by the ultrasonication method for 1 h. Water temperature in the bath was kept below 35 °C by adding cold water during the process of ultrasonication. Then the suspension was centrifuged at 4000 rpm for 10 min. The supernatant was decanted into an Erlenmeyer flask which had been drying to a constant weight in an oven and the soil was extracted in three times. All of the supernatant was combined and completely evaporated at 65 °C water bath in the hood. After that, the amount of the extractant was determined gravimetrically. All experiments were performed in triplicates. In the meantime, the moisture content of each composite soil sample was determined by weighing approximately 1 g of the moist soil and laying in an oven at 105 °C for 24 h. Recovery of TPH in the experiment by this method ranged from 87.60% to 95.87%.

Initial and final petroleum hydrocarbon fractions including saturated fraction, aromatics, polar and asphaltenes, were determined by the silica gel column chromatography followed by the gravimetric analysis. About 12 cm of activated silica gel and 1 cm dry sodium sulfate on the top were filled into a glass column (Φ10 mm × 100 mm). Asphaltenes were removed by n-hexane precipitation, then dried and weighed [26]. The supernatant without asphaltenes was loaded onto the activated silica gel column that had been pre-eluted with hexane. About 50 ml n-hexane was added to obtain saturated hydrocarbons. Aromatic hydrocarbons were eluted using a 1:1 dichloromethane/hexane mixture. Finally, 50 ml of 1:1 methanol/acetone was applied to the elution of polar fraction. All fractions were then quantified gravimetrically [27].

2.4. Biomass assay

Plants grown in different concentrations of petroleum contaminated soils were harvested after a 4-month culture in order to further validate the tolerance of *I. balsamina* to petroleum contaminants. Soil samples in the rhizosphere zone, were collected and put into the sterilized plastic bag through shaking to dislodge soils adhering to the roots [28]. The rhizosphere soil samples were stored at 4 °C until further analysis. Plant samples were carefully washed with tap water, completely rinsed with distilled water and wiped out redundant water using filter paper. Root length, fresh weight and stem height of *I. balsamina* were measured, respectively. Harvested plant tissues were dried at 60 °C in an oven upon reaching the constant weight to determine dry weight.

2.5. Microbial counts

Soil samples were collected from planted subplots and from completely unplanted subplots. As depicted above, the soil samples located 3–10 mm away from the roots of *I. balsamina* were stored in the sterilized plastic bag at 4 °C until further microbial counts and labeled for distinguishing them. The viable microbial population including bacteria, fungi and actinomycetes was enumerated by the pour plate method [29]. The serial dilution method was employed to obtain three appropriate dilutions for plating. One gram of soil sample was added into 100 ml sterile distilled water with several glass beads to achieve well mixed 10^{-2} dilution. Then 1 ml of 10^{-2} dilution was pipetted into 9 ml sterile distilled water until achieving subsequent dilutions, up to 10^{-5}. Each 1 ml of three dilutions (10^{-3}, 10^{-4}, and 10^{-5} dilution) was pipetted, in triplicate, into a labeled sterile empty Petri plate with the diameter of 9 cm. Aseptically about 15 ml of selected melted agar media, which has been cooled to ≤45 °C, was poured into inoculated Petri dishes and was immediately swirled to mix completely to get a homogenous distribution of microorganisms within the medium. Petri dishes were kept flat on the bench for about 10 min to allow the agar to completely gel. Then all Petri dishes were inverted and incubated at appropriate temperature for a reasonable time sufficient to allow visible colony formation. At the end, the microbial population was enumerated and reported in the colony-forming units (CFU) per gram of dry soil. All experiments were conducted on the bench which has been sterilized using UV light for half an hour.

Bacteria were determined on beef extract–peptone agar following the components: beef extractant, 3.0 g; peptone, 10.0 g; sodium chloride, 5.0 g; agar, 20 g and distilled water, 1000 ml (pH 7.0). The Petri plates were incubated at 37.0 °C for 24 h. Then the bacterial colonies were enumerated and reported in CFU/g dry soil. As for fungi, they were incubated at 25.5 °C for 4 days on the Czapek Dox agar involving various ingredients: sucrose, 30.0 g; sodium nitrate, 2.0 g; potassium chloride, 1.0 g; magnesium sulfate heptahydrate, 0.5 g; iron(II) sulfate heptahydrate, 0.01 g; dipotassium hydrogen phosphate, 1.0 g; agar, 20.0 g; and distilled water, 1000 ml. The pH value was adjusted to the range from 5.0 to 5.5 through adding several drops of 80% lactic acid. After a 4-day incubation, the fungal colonies were enumerated and reported in CFU/g dry soil.

For actinomycetes, starch agar medium was used for incubation containing various components: soluble starch, 20.0 g; potassium nitrate, 1.0 g; dipotassium hydrogen phosphate, 0.5 g; magnesium sulfate heptahydrate, 0.5 g; sodium chloride, 0.5 g; iron(II) sulfate heptahydrate, 0.01 g; agar, 20.0 g and distilled water, 1000 ml (pH 7.2–7.4). Several drops of 5% phenol were added into the medium to inhibit the growth of bacteria. All the Petri plates were incubated at 25.7 °C for 7 days. Then the actinomycetic colonies were enumerated and reported in CFU/g dry soil.

2.6. Statistical analysis

Data were processed with the Microsoft Excel software, Origin 8.0 and SPSS 16.0 on the computer. All the experiments were performed in triplicate in order to decrease the experimental errors. Values were reported as mean ± standard deviation (S.D.). Data were analyzed by one-way ANOVA with the Duncan's multiple range tests to separate means. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Promoted biodegradation of TPHs by I. balsamina

The biodegradation of TPHs in soil planted with *I. balsamina* was significantly higher ($p < 0.05$) than that in their unplanted controls (Fig. 1). TPH degradation by *I. balsamina* in soils with 5000, 10,000, 20,000 and 40,000 mg/kg of PHCs ranged from 65.03% to 18.13% whereas that under their corresponding controls (natu-

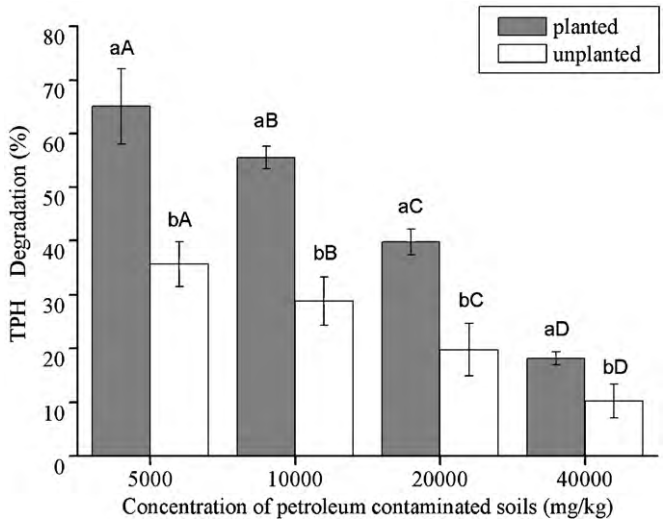

Fig. 1. TPH degradation in *Impatiens balsamina* L. planted soils compared with that under their unplanted controls. Data are mean values with standard deviation. Error bars represent the standard deviation. The means within same TPH treatments followed by the same letter (a and b) were not significantly different at $p < 0.05$. The means among different TPH treatments followed by the same letter (A–D) were not significantly different at $p < 0.05$ either.

ral attenuation) was only in the range from 35.61% to 10.20%. In 10,000 mg/kg of PHC contaminated soil the average degradation of TPHs by the plant was up to 55.50%, whereas that in its corresponding treatment was merely 28.83%. Additionally, reduction of TPHs in soils with 20,000 mg/kg of PHCs was 39.75%, significantly higher ($p < 0.05$) than that (only 10.20%) by natural attenuation. Moreover our results showed TPH degradation by the plant in 5000, 10,000, 20,000 and 40,000 mg/kg of PHC contaminated soils was approximately 1.83, 1.93, 2.01 and 1.78 times more than that by natural attenuation.

The promoted biodegradation of TPHs by *I. balsamina* could be explained by the rhizosphere effect. *I. balsamina* has a strong fibrous root system [22]. In general, due to their fibrous root system plants such as grasses could offer some advantages for promoting biodegradation of organic pollutants [23]. In comparison with the taproot system they can occupy a large volume of soils and grow relatively close to the soil surfaces that are more favorable to establish a strong rhizosphere through the soil. In our present study, several main aspects of *I. balsamina* contribution to enhance biodegradation of TPHs should be assumed. Firstly, in the planted soils the presence of *I. balsamina* may greatly enrich a rhizosphere microbial community by offering exudates and oxygen through its roots. Secondly, roots can also create pores which can improve connectivity and diffusivity of the soil [30], thus offering enhanced water infiltration and a superior environment conducive to degrade pollutants. This further suggested that the presence of *I. balsamina* fibrous roots may induce transpiration-driven mass flow towards the rhizosphere, delivering dissolved pollutants to sites of increased microbial activity and accordingly promote TPH degradation in contrast to their unplanted treatments. Thirdly, plants could release a number of enzymes such as laccases, nitrilases and peroxidases [7] into contaminated soils which could directly degrade organic pollutants. Whereas TPH degradation in the unplanted controls could take place only by volatilization, eluviation, photooxidation and its original microbial activity. On the other hand, due to the penetration of *I. balsamina* fibrous roots through the soil, volatilization and eluviation should be relatively reduced compared to unplanted treatments. As a result, in planted soils *I. balsamina* contribution to TPH biodegradation proves more prominent. Therefore in our experiment the significantly promoted biodegradation of TPHs under the presence of *I. balsamina* and associated stimulated microbial activity could be expected when compared with that by natural attenuation.

Various concentrations of PHCs in soils had different influences on the effectiveness of phytoremediation using *I. balsamina* (Fig. 1). The maximum degradation (65.03%) of TPHs by *I. balsamina* happened in soil contaminated with 5000 mg/kg of PHCs. However, TPH degradation by the plant significantly ($p < 0.05$) reduced with an increase in the concentration of PHCs in soils. It might be attributed to the phytotoxicity of extremely high concentrations of PHCs to the plant and associated microorganisms, thus leading to the low efficacy of phytoremediation especially in the 20,000 and 40,000 mg/kg (only 39.75% and 18.13% TPH degradation) of PHC contaminated soils. However, our results still showed that phytoremediation of petroleum contaminated soils by *I. balsamina* was significantly effective, particularly in soils with 5000 and 10,000 mg/kg of PHCs.

3.2. Tolerance of *I. balsamina* to TPHs

To further evaluate the tolerance of *I. balsamina* to petroleum polluted soils, *I. balsamina* was planted in soils with a series of concentration gradient treatments. After the maturation of *I. balsamina*, quantitative and qualitative plant parameters were determined and recorded, including root length, plant height, shoot fresh weight and dry weight (Fig. 2). Root growth parameters were most useful as an indicator of tolerance of the plant to contaminated soils. In the experiment the root length of *I. balsamina* growing in soils polluted with 5000 and 10,000 mg/kg of PHCs was insignificantly ($p > 0.05$) shorter than that of *I. balsamina* planted in the clean treatment. This result is supported by the previous work of Bossert and Bartha [16], who reported the negative effects of PHCs on plants, yet low level of PHCs (<10,000 mg/kg) does not inhibit plant growth. However, root length parameters in soils with 20,000 and 40,000 mg/kg of PHC contaminated soils were significantly ($p < 0.05$) reduced when compared with that in soils polluted with less than 10,000 mg/kg of PHCs. Obviously, extremely high PHCs might suppress the growth of *I. balsamina*.

Fig. 2 shows the reduction of both the height and weight of *I. balsamina* plants, which were planted in 5000 mg/kg, was insignificant ($p > 0.05$) when compared with that in clean soil. However, most of the tested ornamentals (*Rudbeckia hybrida*, *Dimorphotheca sinuata*, *Cosmos sulphureus*, *Matthiola incana* and *Dianthus chinensis*) died when the concentration of PHCs was up to 5000 mg/kg. Though there was a slight reduction of biomass in soil with a high concentration (20,000 mg/kg) of PHCs, *I. balsamina* still grew well. During the experiment some chlorosis was found in few bottom leaves. In our experiment, the observed symptoms of chlorosis may be the result of PHC stress at the tissue and cellular level of *I. balsamina*. This observation is supported by Alkio et al. [31] who reported chlorosis and even worse necrosis because of a localised H_2O_2 production, oxidative stress and cell death in *Arabidopsis thaliana* exposed to phenanthrene. However, no necrosis has been found in our study, indicating the relatively strong tolerance of *I. balsamina* to TPHs.

Surprisingly in soil contaminated with 40,000 mg/kg of PHCs *I. balsamina* was still alive, though the plant caused an extreme postponement of growth probably due to the high phytotoxicity of extremely high TPHs (4%). This further suggested that the phytotoxicity of extremely high concentrations of PHCs might be a great limitation to the use of *I. balsamina* in the remediation of petroleum contaminated soils. Therefore more steps should be taken into account, such as better agronomic performance of the plants, inoculation with appropriate microbial consortiums to help plants grow healthier and become more tolerant especially in extremely high polluted soils, thus probably improving phytore-

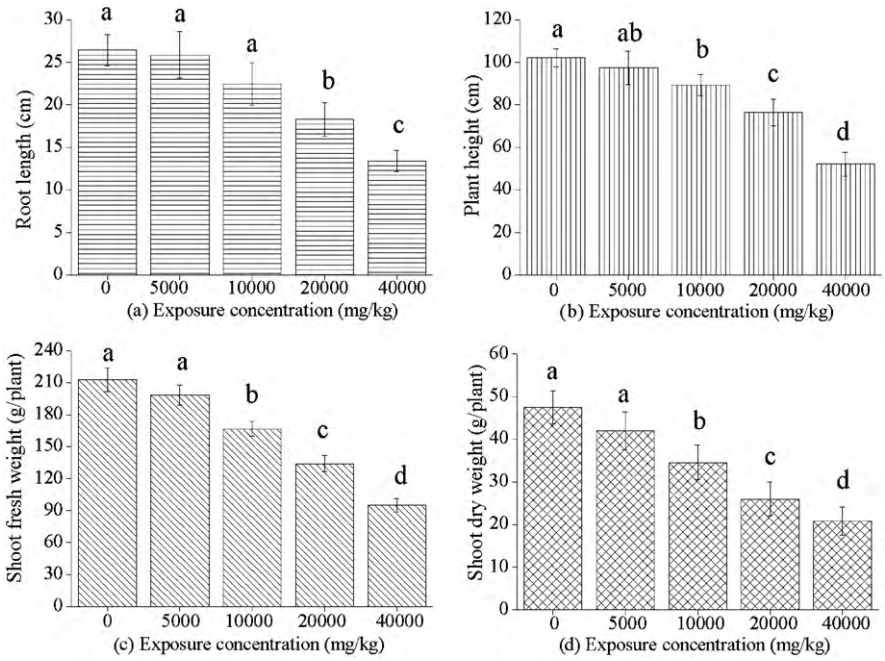

Fig. 2. Biomass including root length (a), plant height (b), shoot fresh weight (c), and shoot dry weight (d) of *Impatiens balsamina* L. under the stress of petroleum contaminated soil. Data are means with standard deviation. Error bars represent the standard deviation. The means among different TPH treatments followed by the same letter (a–d) were not significantly different at $p < 0.05$.

mediation. As discussed above, we may conclude that *I. balsamina* had a good tolerance to 5000 and 10,000 mg/kg of PHCs in soils, leading to the assumption that *I. balsamina* might be appropriate to phytoremedy contaminated soil with less than 10,000 mg/kg of PHCs.

3.3. Changes in petroleum components after phytoremediation

After the 4-month plot-culture experiment, the degradation of saturated hydrocarbons was significantly ($p < 0.05$) higher than that of aromatic, asphaltene and polar fractions wherever in planted or unplanted soils (Fig. 3). Similar results have been reported by Prince [32], who found that saturated hydrocarbons are less phytotoxic and more biodegradable than aromatic and polar fractions. Furthermore the presence of *I. balsamina* significantly improves the biodegradation of saturated hydrocarbons when compared with those in unplanted soils. However, due to the toxicity of PHCs with very high concentrations (2% and 4%) to the growth of *I. balsamina* and associated microorganisms, the degradation of saturated fractions was much lower when compared with that in the relatively serious (0.5% and 1%) polluted soils.

In our research aromatics are more easily biodegradable as compared to the asphaltene and polar fractions (Fig. 3). Results from our research may be explained by Panichayupakaranant et al. [33] who reported *I. balsamina* root cultures could biosynthesize coumarins. It is well established that flavonoids and coumarins comprise one important group of plant compounds in rhizosphere [34]. But few are accumulated in soils, for they can be consumed by soil microorganisms. Moreover the structure of these compounds is similar to many organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) leading to the assumption that the presence of *I. balsamina* may greatly enhance degradation of aromatics. On the other hand, due to their hydrophobic characters and poorer bioavailability asphaltene and polar fractions are more resistant to biodegradation. Therefore, our present results that there was greater reduction of aromatic fractions than asphaltene and polar fractions are expectable.The most remarkable change observed in the experiment was a significant ($p < 0.05$) increase in the asphaltene and polar fractions in planted soil with 20,000 (7.8% increase) and 40,000 (2.4% increase) mg/kg of PHCs. Similarly, Chaîneau et al. [35] has also found an increase in the polar fraction in the soil. Both results reported by Chaîneau et al. and found in our experiment might be explained as below. Usually a temporary or permanent increase in the asphaltene and polar fraction is observed in batch liquid cultures as a result of the accumulation of unassimilated metabolic intermediates such as extended polyaromatics, naphthenic acids, polyhydric phenols and fatty acids during the process

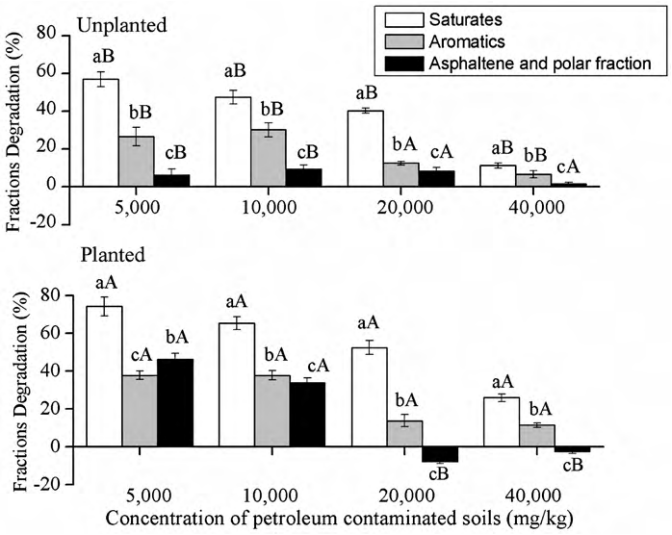

Fig. 3. The degradation of various petroleum fractions both in the *Impatiens balsamina* L. planted soils and the unplanted controls. Data are mean values with standard deviation. Error bars represent the standard deviation of all treatments. The means within same TPH concentration of same treatment (planted/unplanted) followed by the same letter (a–c) were not significantly different at $p < 0.05$. The means among different treatment (planted/unplanted) of same TPH concentration followed by the same letter (A and B) were not significantly different at $p < 0.05$ either.

of biodegrading PHCs [36–38]. Based on the explanation, we might assume that the degradation of saturated fraction and aromatics was enhanced due to the presence of *I. balsamina* in comparison with that of corresponding controls. More oxygen is added into soil through *I. balsamina* root systems than root-free soils. Most of the saturated and aromatic fractions in the synergism between *I. balsamina* rhizosphere and microorganisms would be transformed into ones containing oxygen such as phenols, alcohols, ketones, aldehydes and carboxylic acids (polar compounds), leading to the accumulation of the polar fraction. However, in unplanted soil with 20,000 and 40,000 mg/kg of PHCs, no increase in the asphaltene and polar fraction was found, suggesting that less/no saturated and aromatic fractions be transformed to asphaltene and/or polar fractions. In addition, the phytotoxicity of PHCs to *I. balsamina* was intensified and the activity of the rhizosphere microorganisms was inhibited in soils with 20,000 and 40,000 mg/kg of PHCs (extremely high PHCs) when compared with that in 5000 and 10,000 mg/kg PHC contaminated soils. Accordingly, it might be difficult for *I. balsamina* and associated microorganisms to degrade the original/newly induced polar fraction in soils. Thus, in planted soils we can also observe a significant increase of polar fractions in 20,000 and 40,000 mg/kg PHC polluted soils as compared to that in contaminated soils with 5000 and 10,000 mg/kg of PHCs. It has been reported that a 15–20% maximum biodegradation of asphaltene and polar fractions could occur in optimal cultures [36]. However, this value is much lower with respect to that (46.3%) in our phytoremediation study, showing the effective phytoremediation of our plant. On the whole, the results indicated that *I. balsamina* might have the potential for effective phytoremediation of contaminated soils with ≤10,000 mg/kg of PHCs.

3.4. Effects on microbial population

The total number of bacteria, fungi and actinomycete was evaluated and enumerated after phytoremediation (Fig. 4). The number of the three microorganisms decreased with an increase in the concentration of PHCs in contaminated soils planted with *I. balsamina*. It showed that the toxicity of PHCs depressed the activity and the proliferation of rhizosphere microorganisms. Especially in the soil with 40,000 mg/kg of PHCs, the number of living microorganisms extremely decreased.

The amount of bacteria and fungi in the clean soil was 2.93×10^5 and 2.00×10^4 CFU/g dry soil, respectively. Both the number of bacteria and fungi increased in the soils with 5000 and 10,000 mg/kg of PHCs, respectively, compared with that in the clean soil. Moreover, the bacterial number was always greater than that in the clean soil, but not the fungal number when the concentration of PHCs is equal to and higher than 20,000 mg/kg. The results can be explained that bacteria and fungi in relatively mild polluted soils (5000 and 10,000 mg/kg) could be adaptive to the surrounding environment and used PHCs as a carbon source to proliferate as well as the root exudates excreted by *I. balsamina*. Thereby, the number increased comparatively. These results further showed that the rhizosphere bacteria and fungi were probably responsible for the biodegradation of TPHs, which is consistent with the results reported by Kästner et al. [39]. Hence we could deem bacteria and fungi as the dominant population in the contaminated soils when the concentration of PHCs ≤20,000 mg/kg. When it came to actinomycete, the number was always smaller than that in the clean soil. The decrease tendency of actinomycete suggested that actinomycete was restrained by the toxicity of PHCs and could have little relation to the degradation of TPHs.

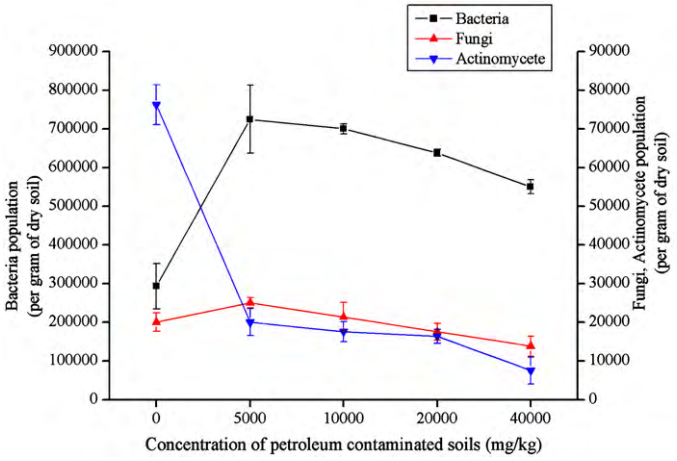

Fig. 4. Effects of petroleum hydrocarbons on the population of bacteria, fungi and actinomycete in rhizosphere soils. Data were presented on graphs with means and standard deviation (S.D.). Error bars represent the standard deviation of three microorganisms.

Table 2
Matrix of correlation coefficients for the TPH degradation, the rhizosphere microbes and root length in the *Impatiens balsamina* L. planted soils.

	TPH degradation	Total rhizosphere microbes	Root length
TPH degradation	1	0.848*	0.904*
Total rhizosphere microbes		1	0.955*
Root length			1

* Significant at 0.05 probability ($p = 0.05$).

Rhizodegradation was responsible for the enhanced removal of PHCs [14]. As an additional compartment, plant roots can interact with both microbes and organic pollutants [16]. Root proliferation of the plant can support a flourishing microbial consortium, thus accelerating biodegradation of PHCs. In turn the healthier microbial consortium can benefit better growth of the plant, thus improving phytoremediation efficiency. However, due to the complexity of rhizosphere, more information about relationships between *I. balsamina* root–microbial interactions in respect to TPH degradation needs to be further extracted, which is pointed in Table 2. Table 2 shows the correlation coefficients among TPH degradation, the total rhizosphere microbes and the root length of *I. balsamina* by the partial correlation coefficient. Degradation of TPHs was significantly ($r = 0.904$, $p < 0.05$) related to the root length of *I. balsamina*. The high positive correlation ($r = 0.848$, $p < 0.05$) was also found between TPH degradation and the total rhizosphere microbes. The significant ($r = 0.955$, $p < 0.05$) positive correlation between the total rhizosphere microbes and the root length of *I. balsamina* was also found. These results further indicated that the total rhizosphere microbes and the growth of *I. balsamina* matched fairly well with TPH degradation.

4. Conclusions

The presence of *I. balsamina* promoted the dissipation of TPHs in soils. After the seed-maturation of *I. Balsamina*, the residual levels of TPHs in planted soils were significantly lower ($p > 0.05$) than that in unvegetated soils. The average degradation of TPHs by *I. Balsamina* in 5000 and 10,000 mg/kg PHC contaminated soils was 65.03% and 55.50%, respectively, while in their corresponding controls (natural attenuation) the reduction of TPHs was only 35.61% and 28.83%, respectively. Moreover, the reduction of TPHs in the polluted soils with ≤20,000 mg/kg of PHCs by *I. balsamina* associated with the rhizosphere microorganisms proved higher than that in the soils with extremely high concentrations of PHCs. Saturated hydrocarbons are the most easily biodegradable and have the highest degradation among various petroleum fractions. Especially

in the vegetated soils with 5000 and 10,000 mg/kg of PHCs, the reduction of saturated hydrocarbons was up to 74.31% and 65.30%, respectively. The release of polar metabolic byproducts during phytoremediation of contaminated soils with ≥20,000 mg/kg of PHCs by *I. balsamina* may occur. To acquire a possible positive feedback on the overall phytoremediation process, more time is desirable for phytoremediation of contaminated soils with high/extremely high PHCs using *I. balsamina.* Appropriate microbial inocula may accelerate the biodegradation of these polar metabolic byproducts in the root zones. Results also indicated that *I. balsamina* had a strong tolerance to the contaminated soils with ≤10,000 mg/kg of PHCs. Both the bacterial and fungal number in vegetated soils with 5000 and 10,000 mg/kg of PHCs were greater than that in the clean soil. The soil bacteria and fungi associated with *I. balsamina* were probably responsible for the enhanced removal of TPHs from contaminated soils. The high correlations ($r = 0.848–0.955$) were found among TPH degradation, the rhizosphere microbes, and the root length of *I. balsamina.* Based on the results, *I. balsamina* L. might be considered as a potential ornamental plant for effective phytoremediation of ≤10,000 mg/kg petroleum contaminated soils, although mechanisms and toxicological risk associated with phytoremediation and temporary or permanent increase of the polar fraction should be subjected to further investigation.

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References

[1] R. Margesin, D. Labbé, F. Schinner, C.W. Greer, L.G. Whyte, Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils, Appl. Environ. Microbiol. 69 (2003) 3085–3092.
[2] Energy Information Administration, US Department of Energy, The International Energy Outlook 2010, Report No. DOE/EIA-0484(2010). Available from: http://www.eia.doe.gov/oiaf/ieo/index.html.
[3] R.B. Meagher, Phytoremediation of toxic elemental and organic pollutants, Curr. Opin. Plant Biol. 3 (2000) 153–162.
[4] Q. Chaudhry, M. Blom-Zandstra, S. Gupta, E.J. Joner, Utilizing the synergy between plants and rhizosphere microorganisms to enhance breakdown of organic pollutants in the environment, Environ. Sci. Pollut. Res. 12 (2005) 34–48.
[5] K. Euliss, C.H. Ho, A.P. Schwab, S. Rock, A.K. Banks, Greenhouse and field assessment of phytoremediation for petroleum contaminants in a riparian zone, Bioresour. Technol. 99 (2008) 1961–1971.
[6] J.L. Kirk, J.N. klironomos, H. Lee, J.T. Trevors, The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil, Environ. Pollut. 133 (2005) 455–465.
[7] Q.X. Zhou, Y.F. Song, Principles and Methods of Contaminated Soil Remediation, Science Press, Beijing, China, 2004.
[8] J. Gurska, W.X. Wang, K.E. Gerhardt, A.M. Khalid, D.M. Isherwood, X.D. Huang, B.R. Glick, B.M. Greenberg, Three year field test of a plant growth promoting rhizobacteria enhanced phytoremediation system at a land farm for treatment of hydrocarbon waste, Environ. Sci. Technol. 43 (2009) 4472–4479.
[9] S.H. Wei, Q.X. Zhou, Phytoremediation of cadmium-contaminated soils by *Rorippa globosa* using two-phase planting, Environ. Sci. Pollut. Res. 13 (2006) 151–155.
[10] A.C. Dietz, J.L. Schnoor, Advances in phytoremediation, Environ. Health Perspect. 109 (2001) 163–168.
[11] E. Escalante-Espinosa, M.E. Gallegos-Martínez, E. Favela-Torres, M. Gutiérrez-Rojas, Improvement of the hydrocarbon phytoremediation rate by *Cyperus laxus* Lam. inoculated with a microbial consortium in a model system, Chemosphere 59 (2005) 405–413.
[12] M.R.T. Palmroth, J. Pichtel, J.A. Puhakka, Phytoremediation of subartic soil contaminated with diesel fuel, Bioresour. Technol. 84 (2002) 221–228.
[13] T.A. Anderson, E.A. Guthrie, B.T. Walton, Bioremediation in the rhizosphere, Environ. Sci. Technol. 27 (1993) 2630–2636.
[14] A.P. Schwab, M.K. Banks, Biologically Mediated Dissipation of Polyaromatic Hydrocarbons in the Root Zone, Bioremediation through Rhizosphere Technology, American Chemical Society, Washington, DC, 1994, pp. 363–375.
[15] J.B. Culbertson, I. Valiela, M. Pickart, E.E. Peacock, C.M. Reddy, Long-term consequences of residual petroleum on salt marsh grass, J. Appl. Ecol. 45 (2008) 1284–1292.
[16] I. Bossert, R. Bartha, The fate of petroleum in soil ecosystems, in: R.M. Atlas (Ed.), Petroleum Microbiology, Macmillan, New York, 1984, pp. 435–474.
[17] H.H. Liste, D. Felgentreu, Crop growth, culturable bacteria, and degradation of petrol hydrocarbons (PHCs) in a long-term contaminated field soil, Appl. Soil Ecol. 31 (2006) 43–52.
[18] S.S. Radwan, H. Al-Awadhi, I.M. El-Nemr, Cropping as a phytoremediation practice for oily desert soil with reference to crop safety as food, Int. J. Phytorem. 24 (2000) 383–396.
[19] J.N. Liu, Q.X. Zhou, T. Sun, L.Q. Ma, S. Wang, Growth responses of three ornamental plants to Cd and Cd–Pb stress and their metal accumulation characteristics, J. Hazard. Mater. 151 (2008) 261–267.
[20] X.F. Wang, Q.X. Zhou, Ecotoxicological effects of cadmium on three ornamental plants, Chemosphere 60 (2005) 16–21.
[21] X.F. Wang, Resource potential analysis of ornamentals applied in contaminated soil remediation, A dissertation in Graduate School of Chinese Academy of Sciences, Beijing, 2005.
[22] Y.W. Zheng, D.Y. Hong, Flora of China, vol. 12, Science Press, Beijing, 2009.
[23] Th. Günther, U. Dornberger, W. Fritsche, Effect of ryegrass on biodegradation of hydrocarbons in soil, Chemosphere 33 (1996) 203–215.
[24] US EPA Method 3550c, Ultrasonic Extraction, United States Environmental Protection Agency, SW-846 Manual, U.S. Government Printing Office, Washington, DC, 2007. Available from: http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/3550c.pdf.
[25] US EPA Method 1664, Oil and Grease Analysis in Wastewater, United States Environmental Protection Agency, SW-846 Manual, U.S. Government Printing Office, Washington, DC, 2007. Available from: http://www.epa.gov/waterscience/methods/method/oil/.
[26] M.V. Kök, Ö. Karacan, R. Pamir, Kinetic analysis of oxidation behavior of crude oil SARA constituents, Energy Fuels 12 (1998) 580–588.
[27] S. Mishra, J. Jyot, R.C. Kuhad, B. Lal, In situ bioremediation potential of an oily sludge-degrading bacterial consortium, Curr. Microbiol. 43 (2001) 328–335.
[28] L.A. Phillips, C.W. Greer, J.J. Germida, Culture-based and culture-independent assessment of the impact of mixed and single plant treatments on rhizosphere microbial communities in hydrocarbon contaminated flare-pit soil, Soil Biol. Biochem. 38 (2006) 2823–2833.
[29] Q.M. Zhang, H.Y. Li, L. Wang, Experimental Method in Study of Environmental Biology, 1st edition, Chemical Industry Press, Beijing, 2005.
[30] I.M. Young, J.W. Crawford, Interactions and self-organization in the soil–microbe complex, Science 304 (2004) 1634–1637.
[31] M. Alkio, T.M. Tabuchi, X.C. Wang, A. Colon-Carmona, Stress responses to polycyclic aromatic hydrocarbons in *Arabidopsis* include growth inhibition and hypersensitive response-like symptoms, J. Exp. Bot. 56 (2005) 2983–2994.
[32] R.C. Prince, Bioremediation: an overview of how microbiological processes can be applied to the cleanup of organic and inorganic environmental pollutants, in: Encyclopedia of Environmental Microbiology, John Wiley & Sons, 2002.
[33] P. Panichayupakaranant, H. Noguchi, W. De-Eknamkul, U. Sankawa, Naphthoquinones and coumarins from *Impatiens balsamina* root cultures, Phytochemistry 40 (1995) 1141–1143.
[34] A.C. Singer, I.P. Thompson, M.J. Bailey, The tritrophic trinity: a source of pollutant-degrading enzymes and its implications for phytoremediation, Curr. Opin. Microbiol. 7 (2004) 239–244.
[35] C.H. Chaîneau, G. Rougeux, C. Yéprémian, J. Oudot, Effects of nutrient concentration on the biodegradation of crude oil and associated microbial populations in the soil, Soil Biol. Biochem. 37 (2005) 1490–1497.
[36] J. Oudot, J. Dupont, S. Haloui, M.F. Roquebert, Biodegradation potential of hydrocarbon-assimilating tropical fungi, Soil Biol. Biochem. 25 (1987) 1167–1173.
[37] F. Chaillan, A.Le. Flèche, E. Bury, Y. Phantavong, P. Grimont, A. Saliot, J. Oudot, Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms, Res. Microbiol. 155 (2004) 587–595.
[38] K. Suguira, M. Ishihara, T. Shimauchi, S. Harayama, Physicochemical properties and biodegradability of crude oil, Environ. Sci. Technol. 31 (1997) 45–51.
[39] M. Kästner, M. Breuer-Jammali, B. Mahro, Enumeration and characterization of the soil microflora from hydrocarbon-contaminated soil sites able to mineralize polycyclic aromatic hydrocarbons (PAH), Appl. Microbiol. Biotechnol. 41 (1994) 267–273.