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Bioremediation potential of a phenol degrading bacterium, *Rhodococcus erythropolis* SKO-1

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Biodegradation of phenol is a major focus of toxic organic compound degradation by microorganisms isolated from polluted areas. An increasing number of bacteria and fungi possessing unique biodegradation capabilities have been isolated in recent years. In this study a new isolate, *Rhodococcus erythropolis* SKO-1, from polluted soils in the Tehran oil refinery region, is reported. Identification was performed by sequencing of 16S rDNA and confirmed by morphological and biochemical characterization. Phenol was consumed as the sole carbon source, and the ortho pathway was confirmed as the metabolic route of phenol biodegradation by the isolate. After adaptation, phenol degradation was approximately 99.64% of an initial concentration of 1000 mg phenol l^{-1} in 56 h. Enrichment of minimal salt medium by addition of yeast extract resulted in further phenol tolerance and increased phenol degradation capacity up to 1200 mg phenol l^{-1}. The most efficient conditions for biodegradation were obtained at 30°C. The promising results indicate that the strain could be used in bioremediation of phenol-containing wastes. © 2011 Progress in Biological Sciences, Vol. 1, No. 1, 31-40.

KEY WORDS: Biodegradation, contaminated soil, monoaromatic hydrocarbons, oil refinery, phenol, *Rhodococcus erythropolis*.

INTRODUCTION

Since the beginning of the industrial revolution, an enormous number of organic compounds have been chemically synthesized, many of which are subsequently released as waste (Fernando and Aust, 1994). Research has long been directed at the elimination of the wastes; however, in practice, only two procedures have been utilized: conversion of chemical substances to release of new products (eventually converted to new waste) or compaction of chemicals into smaller volumes and burying the concentrated compounds in underground. Distribution of CO_2 in the atmosphere, which is produced by burning of waste materials and burying the ash from incineration of dried active sludge are two of the more common examples. Rapid conversion of toxic and hazardous materials into less harmful ones is a necessity.

Phenol is one of the most common toxic environmental pollutants (Chung et al., 1998; Wei et al., 2008) and mainly originates from industrial processes such as refineries (6-500 mg l^{-1}), coking operations (28-3900 mg l^{-1}), coal processing (9-6800 mg l^{-1}), and manufacture of petrochemicals (2.8-1220 mg l^{-1}). Other sources of wastewater containing phenols are pharmaceuticals, plastics, wood products, and paint and paper industries (0.1-1600 mg l^{-1}) (Busca et al., 2008). Phenol is toxic to aquatic life and acts as a substrate inhibitor in biotransformation (Contreras et al., 2008). The bioaccumulation of phenol in human and animal tissue and biomagnification in food chains can lead to severe contamination and hence is a major concern (Dos Santos et al., 2009).

Phenolic wastes are treated by physicochemical methods such as, adsorption, reverse osmosis, electrolytic oxidation, and photo catalysis. These methods are impractical due to high costs and the production of other toxic end products, e.g., phenol conversion into Chlorophenol when chlorination is used (Marrot et al., 2006).

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Bioremediation has developed into an important economical method of waste remediation. Biological removal has become a favored alternative because of lower costs and the possibility of complete mineralization (Singleton, 1994).

Isolation of efficient indigenous phenol degrading microorganisms from phenolic wastes and their cultivation and reuse for bioaugmentation has been used in an attempt to accelerate bioremediation in treatment systems. In addition, exogenous phenol degrading microorganisms can be used in tertiary wastewater treatment procedures, such as biofilteration.

The focus on microbial degradation of phenols in recent years has resulted in the isolation, culture, adaptation, and enrichment of a number of microorganisms that can utilize phenol and its derivatives as a sole source of carbon and energy (Gemini et al. 2005; Kobayashi et al. 2007; Dong et al., 2008; Kotresha and Vidyasagar, 2008). Thus, this research was conducted to identify a native isolate with potential applications in enhancement of phenol bioremediation.

MATERIAL AND METHODS

Chemicals

All chemicals used in this study were of the analytical grade commercially available from Merck. Phenol was chromatographic grade (purity 99.5%, Merck).

Growth media

Minimal salt medium (MSM), as the growth medium base, contained defined concentrations of mineral salts (Arutchelvan et al., 2006). In all cases, pH was adjusted to 7.5, and phenol as the sole carbon source was added at 100 mg l^{-1} final concentration, unless otherwise mentioned. To prepare the MSM/phenol, medium base and phenol solution were mixed thoroughly after sterilization. The MSM was solidified as MSM phenol agar by addition of 1.5% agar when necessary. Trypticase soy broth (TSB) was used as nutrient medium to prepare active pre-cultures.

Screening and selection of phenol-degrading bacteria

Petroleum contaminated soil samples from the Tehran oil refinery (Tehran Province, Iran) were collected from the surface layer (0-10 cm) using a soil auger. The soil characteristics were 54.4% clay, 28% silt, and 17.6% sand, using Bouyoucos hydrometer, 1.18% organic matter, and a pH of 8.7 (Rowell, 1994). Ten grams of soil was suspended in 90 ml aliquots of normal saline, with vigorous shaking for 15min. These suspensions were serially diluted and spread on MSM phenol agar plates. After one-week incubation at 30 ± 0.1°C, the well-defined colonies were purified by streaking on agar plates containing the same medium. Isolates were transferred and grown on MSM phenol agar slants and preserved at 4°C for further experimental studies.

Preparation of inocula

Colonies grown on MSM agar plates supplemented with 470 mg l^{-1} phenol (Dos Santos et al., 2009) were used to inoculate 250 ml Erlenmeyer flasks with cotton plugs containing 50 ml of the same liquid medium with 100 mg l^{-1} phenol.

Colonies of the isolate were individually precultured in TSB and incubated in a rotary shaking incubator at 30 ± 0.1°C and 130 rpm to the mid-exponential phase. The bacterial cells were then collected by centrifugation at 8000 rpm for 10 min at room temperature and washed twice with sterilized potassium phosphate buffer (pH 6.8) to eliminate residues of the growth substrate and its metabolites. The resulting pellet was used as initial biomass to inoculate phenol containing MSM for further assessment of phenol degradation. To demonstrate reliability of the tests, control flasks without inoculation were incubated in parallel under the same conditions to ascertain the evaporation losses of phenol.

Acclimatization of culture

The acclimatization of the selected isolate was performed in a phenol containing MSM. Initially, the selected isolate was grown in MSM with 100 mg l^{-1} phenol. Thereafter, cell mass was centrifuged and inoculated into MSM at 200 mg l^{-1}. Incremental additions of phenol to MSM increased concentration to 1000 mg l^{-1}, and the isolate was grown in a shaking incubator at 30 ± 0.1°C and 130 rpm. Samples were withdrawn at

Table 1. Morphological and biochemical characterization of the isolate *Rhodococcus erythropolis* SKO-1.

Characteristic	Result
Morphology	
Configuration	Round
Margin	Entire
Elevation	Convex
Density	translu-cent
Surface	Smooth
Pigment	+
Gram reaction	+
Endospore formation	-
Biochemical activity	
Oxidase	-
Catalase	+
Esculin hydrolysis	+
Urea hydrolysis	+
Acid production from carbohydrates:	
D(+) Sucrose, , D(+) Maltose	+
L(+) Arabinose, D(+) Cellobiose, D(+) Galactose, D(+) Lactose, Inulin, D(+) Mannose, D(+) Melibiose, α- L(-) Rhamnose, D(-) Ribose, D(+) Trehalose, D(+) Xylose-	-

regular intervals and analyzed for phenol degradation and bacterial growth. Effects of temperature on growth and phenol degradation were also investigated. All tests were done in triplicate; hence the results are the arithmetic means of three independent experiments.

Analytical procedure

To measure the biomass, the samples were centrifuged at 8000 rpm for 20 min. The supernatant was used for phenol determination. The biomass was re-suspended in distilled water and absorbance was measured against distilled water as reference at 600 nm (Kumar et al., 2005). Phenol analysis was carried out by measurement of absorbance at a wavelength of 500 nm using a UV-Vis spectrophotometer (Milton Roy, USA) after color development by 4-aminoantipyrine, following the standard methods for the examination of water and wastewater (Greenberg and Eaton, 1998).

Identification of the bacterial isolate

Genomic DNA was extracted by the method of SET buffer (Sambrook et al., 1989). The 16S rRNA gene of the strain was amplified from the bacterial genomic DNA by PCR using universal primers of 27f (5′-GTTTGATCCTGGCTCAG-3′) as forward and 1492r (5′-TACGGTTA CCTTGTTACGACTT-3′) as reverse primers (Heuer et al., 1997). PCR amplification was carried out as follows: 5 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C, plus an additional 5 min cycle at 72°C.

The 16S rDNA sequence of SKO-1 and its related references deposited in GenBank were aligned using Bioedit. A phylogenetic tree was constructed according to the neighbor-joining method (Saitou and Nei, 1987) with MEGA 4.1 software. The dataset was bootstrapped 1000 times. The 16S rDNA sequence of *Nocardia miyunensis* 117T (AY639901) was used as outgroup.

Morphological, biochemical and carbon utilization tests were carried out to confirm the results of molecular approach (Yoon et al., 2000; Duetz et al., 2001; Yoong and Land, 2001; Li et al., 2004; Goodfellow and Maldonado, 2006).

Ring fission mechanism

The ring fission mechanism was revealed using the Rothera reaction, a chemical reaction modified from Kilby (1948). Cells grown on MSM agar plates supplemented with 1mM phenol were suspended in 0.02 M Tris buffer (pH 8). The suspension was toluenized, and 0.2 ml catechol (0.1 M) was added to each 2 ml aliquot of suspension. The samples were shaken for 1 h at 30 ± 0.1°C and then tested for the appearance of β-ketoadipate (indicating an *ortho* cleavage) by the modified Rothera reaction (Palleroni, 2005).

RESULTS

Isolation and identification of the strain

In this study, seven soil samples were diluted and plated repeatedly on MSM phenol agar. Under selective pressure of phenol as the sole source of carbon and energy, only one of five morphotypes could tolerate concentrations of phenol greater than 100 mg l^{-1} and use it as the sole source of carbon and energy. The selected isolate was designated as SKO-1. On the basis of 16S rDNA sequencing (Fig. 1) and morphological and biochemical characterization (Table 1), the strain was found to be closely related to *Rhodococcus erythropolis* and subsequently named *R. erythropolis* SKO-1.

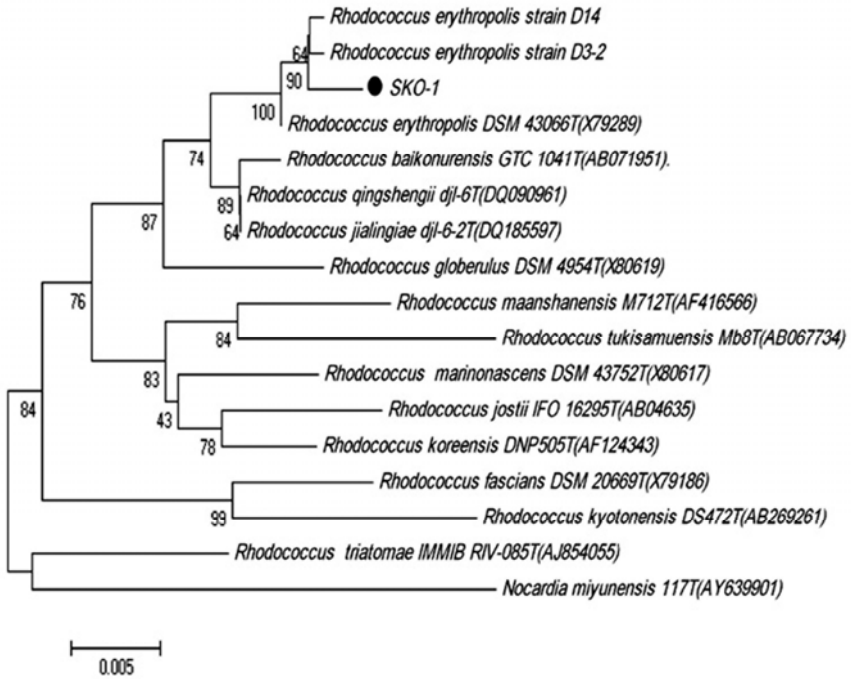

Fig. 1 Phylogenetic analysis based on the 16S rDNA sequences of the phenol-degrading strain SKO-1. Neighbor-joining model is employed for the tree construction, and bootstrap values obtained with 1000 repetitions are indicated as percentages at the nodes.

Acclimatization process

The acclimatization of SKO-1 was carried out as described (Fig. 2). Before acclimatization, the bacterial cells were able to grow and degrade 500 mg l^{-1} phenol within 48 h, while at higher phenol concentrations cell lysis occurred. During the acclimatization process, the bacterial cell became adapted to 600 and 700 mg l^{-1} phenol within 56 h, to 800 and 900 mg l^{-1} phenol within 72 h, and eventually completely degraded 1000 mg l^{-1} phenol within 72 h with no signs of cell lysis.

Enrichment of minimal salt medium by addition of yeast extract resulted in further phenol tolerance and increased degradation capacity up to 1200 mg l^{-1} phenol (Fig. 3). However, this feature was obtained independent of adaptation since it was not observed in the absence of yeast extract, even though previous acclimatization had taken place in the presence of this enrichment agent. In the sterile control, no measurable phenol reduction occurred throughout the experiments, indicating that no physical or chemical removal of phenol occurred in the culture flasks.

Biodegradation capacity and growth after adaptation

As depicted in Fig. 4, the maximum degradation capacity and the influence of the adaptation to the substrate were studied in batch mode within flasks. An increased biodegradation time was observed as the initial phenol concentration was increased. The well-acclimatized culture of *R. erythropolis* SKO-1 degraded the initial phenol concentration of 500 mg l^{-1}, 700 mg l^{-1}, and 1000 mg l^{-1} completely in less than 32, 48, and 72 h, respectively.

Phenol degradation in the well-acclimatized culture of *R. erythropolis* SKO-1 was approximately 99.68%, 99.68%, and 99.64%, for an initial concentration of 500, 700, and 1000 mg l^{-1} phenol in 24, 32, and 56 h, respectively. The non-adapted bacteria did not degrade phenol in 1000 mg l^{-1}, and therefore were unable to survive (Fig. 4).

A comparison of data in Fig. 4 clearly shows the effect of the acclimatization on phenol degradation by *R. erythropolis* SKO-1.

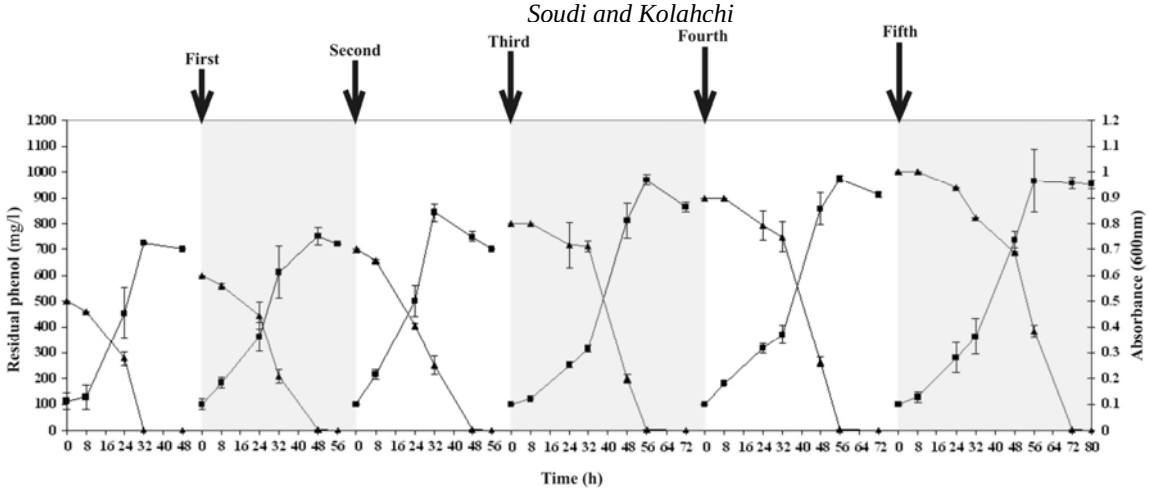

Fig. 2 The cell growth of *Rhodococcus erythropolis* SKO-1 and phenol degradation in the minimal salt medium containing initial 500 mg phenol l^{-1} and step by step transfer of cells into media containing increased concentration of phenol (incremental 100 mg l^{1}), from 600 up to 1000 mg l^{-1} during the acclimatization process. (▲): residual phenol, (■): absorbance due to the cell growth of *Rhodococcus erythropolis* SKO-1. Arrows show the transfer into new culture medium in each step (The similar pattern of adaptation process in range of 100 to 400 mg phenol l^{-1} was not shown).

Mechanism of action

Results demonstrate that *R. erythropolis* SKO-1 is capable of utilizing phenol as a sole carbon source. Results from the Rothera reaction indicate the involvement of an *ortho* metabolic pathway. In microbial degradation of phenol under aerobic conditions, degradation is initiated by oxygenation in which the aromatic ring is initially monohydroxylated by a mono oxygenase phenol hydroxylase at an *ortho* position, to form catechol. This is the main intermediate stage resulting from metabolism of phenol by different microbial strains. Depending on the type of strain, the catechol then undergoes a ring cleavage which can occur either at the *ortho* position, initiating the *ortho* pathway that leads to the formation of succinate and acetyl-CoA, or at the *meta* position, initiating the *meta* pathway that leads to the formation of pyruvate and acetaldehyde (Van Schie and Young, 2000).

In the current study, *R. erythropolis* efficiently degraded 1000 mg l^{-1} phenol at 30 ± 0.1°C, while, at 25°C and 35°C, phenol degradation was not observed.

DISCUSSION

In this study, phenol degrading *R. erythropolis* SKO-1 was isolated under selective pressure of phenol as the sole source of carbon and energy. Obtaining bacteria with a desired metabolic capability directly from natural habitats is rarely feasible. Generally, some form of selection or enrichment is employed to obtain bacteria with a desired metabolic capability, but enrichment may introduce a bias, not only in the organisms, but also in their degradative genes (Marchesi and Weightman, 2003). Due to repeated transfer over long periods under nonselective conditions, laboratory strains may no longer retain their original metabolic capability. Microbial consortia are used when isolation of pure cultures with the desired metabolic capability has not been possible. Biodegradation of many chemicals by microbial consortia has been reported (Dong et al., 2008; Neilson and Allard, 2008).

In this study, the efficient strain was isolated from clay soils. A study of phenol biodegradation by *Pseudomonas stutzeri* showed that not only the capability of the microorganism, but also type of the soil, strongly influences the rate of phenol removal and survival of bacteria (Morzik et al., 2008).

The SKO-1 strain of *R. erythropolis* was isolated from a petroleum refinery site in Tehran. Data supports the prevalence of efficient phenol degrading bacteria in soils polluted with oil, petrochemical wastes, and synthetic phenolics. Banerjee and Ghoshal (2010) isolated two phenol degrading strains of *Bacillus cereus* from similar sites such as petroleum refinery and oil exploration sites. Phenol degrading bacteria

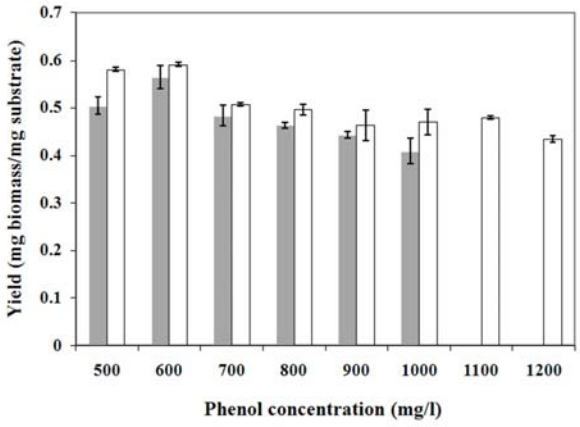

Fig. 3 Yield of *Rhodococcus erythropolis* cell biomass, previously adapted to phenol in the presence of yeast extract. Cells were grown in minimal salt medium (MSM) plus phenol as carbon source in the presence (□) and absence (■) of yeast extract (with 0.06 g l^{-1} concentration). Absence of column indicates complete cell lysis.

have been found in natural soils and sediments (Gemini et al. 2005; Dong et al., 2008) as well as in the intestines of marine animals (Kobayashi et al., 2007). In some cases, closely related strains showed different tolerance and degradation properties (Felföldi et al., 2010). Phenol biodegradation has been studied in detail primarily with *Pseudomonas* species (Powlowski and Shingler, 1994; Gonzàles et al., 2001; Kumar et al., 2005; Kulkarni and Chaudhari, 2006; Afzal et al., 2007; Yang and Lee, 2007; Kotresha and Vidyasagar, 2008; Saravanan et al., 2008). Other microorganisms such as *Phanerochaete chrysosporium* (Pérez et al., 1997), *Rhodococcus* spp. (Margesin et al., 2005), *Candida tropicalis* (Yan et al., 2006), *Candida* sp. (Hu et al., 2006), *Bacillus brevis* (Arutchelvan et al., 2006), *Penicillium chrysogenum* (Leitão et al., 2007), and *Alcaligenes* sp. (Nair et al., 2007) have also been shown to degrade phenol and phenolic compounds.

SKO-1 efficiently degraded phenol under aerobic conditions. Although both aerobic and anaerobic microorganisms are able to degrade phenol, aerobic processes are preferred (Contreras et al., 2008). Strain SKO-1 was closely related to *R. erythropolis*. Among all microorganisms listed as efficiently degrading phenol, the pure culture of strains of *Rhodococcus* spp. were found to be resistant to a number of toxic xenobiotics, and their ability to degrade many of these compounds has been reported (Martínková et al., 2009).

Gonzáles et al. (2001) showed a degradation capacity of more than 90% of 500 mg l^{-1} phenol in 25 h by immobilized cells of *Pseudomonas putida* ATCC 17484. The degrading ability of the bacterium was examined in a range of 200-1000 mg l^{-1}. In contrast to our study, they used only two adaptation steps, and the phenol biodegradation capability of *Pseudomonas putida* ATCC 17484 decreased with increasing phenol concentration. When a phenol concentration of 1000 mg l^{-1} was used, the degradation time decreased from 340 h in the first adaptation to 260 h in the second one while a decrease in the lag phase was also noted. The well-acclimatized culture of *R. erythropolis* SKO-1 degraded initial phenol concentrations of 500 mg l^{-1}, 700 mg l^{-1} and 1000 mg l^{-1} completely in less than 32, 48, and 72 h, respectively. The biodegradation of industrial wastes can be improved if the microorganisms are previously adapted to the toxic chemical. This is much more important when dealing with toxic compounds such as phenol (Zilli, 1993).

Phenol probably exerts its toxic effect by changing membrane function and influences protein to lipid ratios in the membrane (Sikkema et al., 1995). Hence, changes in the membrane fluidity and induction of certain enzymes during acclimatization of *R. erythropolis* appear to be the major responses of the bacterium to the presence of toxic compounds (Heipieper et al., 1994; de Carvalho and de Fonseca, 2005; Kumar et al., 2005).

The presence of yeast extract was reported to enhance the affinity of *Pseudomonas putida* for phenol (Armenante et al., 1995; Lob and Tar, 2000), and the present study showed that the addition of 0.06 g l^{-1} yeast extract increased phenol degradation (Fig. 3). Yeast extract may stimulate the viability of cells and enhance degradation by providing growth factors and by providing a limited number of primary metabolites produced through the phenol biodegradation pathway. Temperature might play an equivalent or larger role than nutrient availability in the degradation of organic pollutants (Margesin and Schinner, 1997). SKO-1

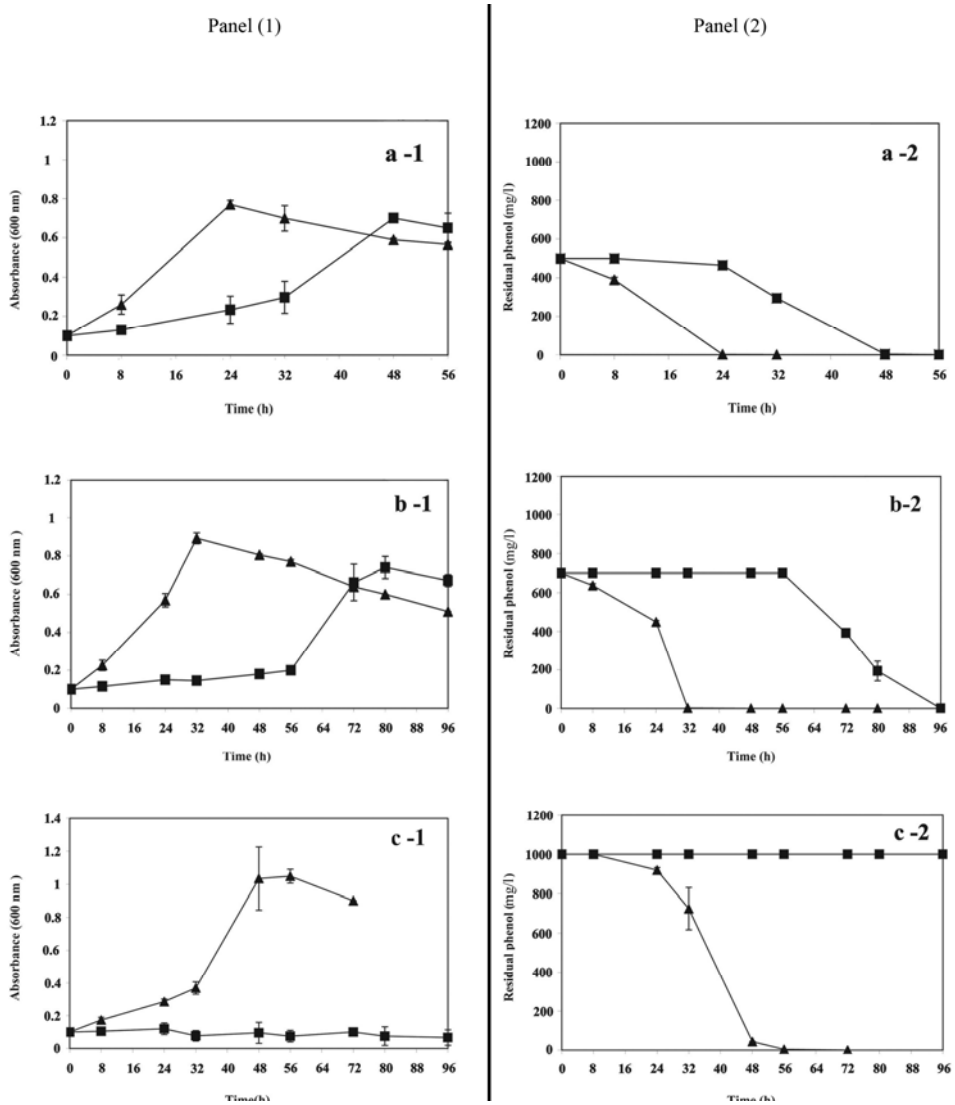

Fig. 4 Influence of adaptation on the phenol degradation capacity and cell growth of *R. erythropolis*. Cell growth (Panel1) and phenol degradation (Panel 2) in 500 mg l^{-1} (a-1, a-2), 700 mg l^{-1} (b-1, b-2) and 1000 mg l^{-1} (c1, c-2); without adaptation (■) and following adaptation process (▲).

showed increased activity at 30°C, and cell lysis occurred at 25 and 35°C. Most laboratory studies of phenol degradation have been carried out at the optimum temperature of 30°C, and the extent and the rate of degradation was shown to be relatively sensitive to deviation outside the optimal range (Mordocco et al., 1999; Annadurai et al., 2002; Paraskevi and Euripide, 2005).

Conclusions

In this study, *Rhodococcus erythropolis* SKO-1 was identified as an efficient strain for phenolbiodegradation. This strain showed a good capacity for adaptation with increased concentra

tions of phenol, and its activity was enhanced in the presence of small amounts of enrichment factors. The results further confirm that the large array of enzymes produced by the species *R. erythropolis* justifies the prospective application of this bacterium in bioremediation biotechnology.

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