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Isolation and characterization of phenol degrading bacteria from Persian Gulf

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Abstract

Phenol and phenol compounds are environmental pollutants present in industrial wastewaters such as, coal tar, oil refineries and petrochemical plants exist. Phenol removal from industrial effluent is extremely important in protection of environment. Recently phenol biodegradation has been considered. Marine bacteria are the most important phenol biodegrader. In this study, the phenol-degrading bacteria from marine environmental samples (soil and water) were isolated from the Persian Gulf. After three passages, the bacterial growth was measured that four bacteria (F6, F10, F13, F16) has the highest rate of growth. Also, these bacteria were able to remove phenol that was measured by absorbance at 272 nm. The hydrophobicity and emulsification activity was measured in all four bacteria. Finally, after a series of biochemical tests, molecular analysis for strong bacteria in degrading phenol, 16S rRNA gene region amplified with primers specific part of the gene was performed. The sequence result of the gene bank and the highest homology (greater than 98%) were identified as species of bacteria. Genus of isolated bacteria was belonging to *Nitratireductor aquimarinus*, *Nitratireductor aquimarius*, *Marine bacterium*, *Pseudomonas stutzeri*.

Key words: Biodegradation, Persian Gulf, Phenol, Marine environment

Introduction

Phenol and phenolic compounds are found as mono-aromatic compounds in various types of environmental site. As major pollutants, their existence in industrial wastewater of oil refineries, and petrochemical and phenol resin industry plants, has been established (Annaduraia, 2002; Koutny, 2003; Xu, 2003). As well as in industrial effluents, phenol and its derivatives are among the most frequently found pollutants in rivers and landfill run-off waters. The U.S. Environmental Protection Agency (EPA) established a phenol concentration of 0.5 mg l^{-1} as the limit for wastewater discharge into natural water bodies and land or municipal sewerage systems. For

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drinking water, EPA has prescribed a guideline concentration of 1 μg l^{-1} (Sal and Boaventura, 2001; Kumar, 2005). Phenolic compounds have traditionally been removed from industrial effluent by costly physicochemical processes, although biological methods have been applied, offering reduced capital and operating costs. For this purpose various phenol-degrading microorganisms, particularly pseudomonads, have been intensively studied in the past (Watanabe, 1998; Wagner, 1999). The first step in aerobic phenol metabolism is hydroxylation to catechol by phenol hydroxylase, and then catechol is metabolized by different strains via either the *ortho*- or *meta*-fission pathway (Neujahr and Gaal, 1973). There are a number of methods for determining maximum phenol degradation and the adherence of these bacteria to surfaces, but these methods are time consuming (Whiteley , 1998; Kahru , 1998). Phenol degrading bacteria mainly investigated in the terrestrial environments and there are a few studies on phenol biodegradation in marine environment. Persian Gulf as one of the world's major oil resources is considered as the most polluted outside territorial waters. This pollution is due to the improper disposal of sewage, industrial waste and the emission of pollutants by power plants and also pollutants caused by oil spills and wastewater from petrochemical industries and so forth. They all enter into the environment. With the growing industrial and chemical activities in the Persian Gulf and increasing pollution caused by these activities in the region, it essentially demands an efficient and economical way to remove these pollutants from the environment. The object of this investigation is to isolate Phenol-degrading bacteria from the coastal area of the province of Bushehr and to identify the superior strain. Subsequently, these strains could be used to prepare a bio pilot for bioremediation.

Materials and Methods

Sampling

For isolation of phenol-degrading bacteria, marine environmental samples were collected from various sites in Persian Gulf. The samples included contaminated seawater and marine sediment. Sediment samples (1 g) were taken from 1-12 cm below the surface using a sterile knife and transferred to phenol broth for enrichment and isolation. Seawater samples were collected from a depth of 15 cm in sterile 1000-ml bottles and transported on ice to the laboratory for isolation on the same day.

Media

A synthetic phenol broth medium used was based on mineral salt medium containing (g L^{-1}): KH_2PO_4, 2.25; K_2HPO_4, 2.25; $(NH_4)_2SO_4$, 1.0; $MgCl_2.6H_2O$, 0.2; NaCl, 4; $FeCl_3.6H_2O$, 0.02; and $CaCl_2$, 0.01; pH= 7. This basal medium was supplemented with phenol as sole carbon source at a concentration of 3 mM (Watanabe et al., 1998). Phenol agar medium was prepared from the phenol broth solidified with 15 g agar l^{-1}. All chemicals were obtained from Merck and Sigma. Media were sterilized at 121°C for 15 min.

Isolation and selection of phenol degrading bacteria

Portions of sediment (1 g) or seawater (1 ml) samples were added to Erlenmeyer flasks containing 100 ml phenol broth and the flasks incubated for 7 days at 30°C on a rotary shaker (INFORS AG) operating at 180 rpm. Then 5-ml aliquots were removed to fresh phenol broth medium (100 ml). After a series of four further subcultures (each after 2 days at 30°C on the shaker), inoculums from

the flasks was streaked out and phenotypically different colonies purified on phenol agar. Phenotypically different colonies obtained from the plates were transferred to fresh mineral agar plates with and without phenol to eliminate autotrophs and agar-utilizing bacteria. The procedure was repeated and isolates only exhibiting pronounced growth on phenol were stored for further characterization.

Growth and phenol removal assay

Growth rate of the isolates were routinely assessed indirectly by turbidity measurement as (O.D. at 600 nm) in a UV-visible spectrophotometer (Shimadzu UV-160, Japan). The amount of phenol removal by bacteria in medium ONR7a versus the control were determined by the UV spectrophotometer at a wavelength of 272nm, after shaking for one week at 30° C with 150 rpm, and the percentage of phenol removal were measured using the following equation (Quintana *et al*., 1997).

$$\text{Percent of phenol removal} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}}$$

Measurement of emulsification activity and bacterial adherence to hydrocarbons (BATH)

The emulsification activity (E24) was determined by combining equal volumes of hexadecane and cell-free culture broth, mixing with a vortex for 2 min and leaving the mixture to stand for 24 h. The emulsification activity was calculated as a percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm). Measurement of the bacterial adhesion to hydrocarbon was performed as described by(Pruthi and Cameotra, 1997).

Molecular identification of robust phenol degrading bacteria

Analysis of 16S rRNA was performed to the taxonomic characterization of isolated strains. Total DNA extraction of bacterial strains was performed with the CTAB method (Winnepenninckx et al., 1993). The bacterial 16S rRNA loci were amplified using the forward domain specific bacteria primer, Bac27_F ($5'$-AGAGTTTGATCCTGGCTCAG-$3'$) and universal reverse primer Uni_1492R ($5'$-TACGYTACCTTGTTACGACTT-$3'$). The amplification reaction was performed in a total volume of 50 µl consisting, 1X solution Q (Qiagen, Hilden, Germany), 1X Qiagen reaction buffer, 1 µM of each forward and reverse primer, 10 µM dNTPs (Gobco, Invitrogen Co, Carlsbad, CA), and 2 U of Qiagen Taq polymerase (Qiagen). Amplification for 35 cycles was performed in a thermacycler GeneAmp 5700 (PE Applied Biosystem, Foster City, CA, USA). The temperature profile for PCR was kept, 95 °C for 5 min (1 cycle); 94 °C for 1 min and 72°C for 2 min (35 cycles), followed by 72 °C for 10 min at the end of final cycle (Troussellier et al. 2005). The 16S amplified was sequenced with a Big Dye terminator V3.1 cycle sequencing kit on an automated capillary sequencer (model 3100 Avant Genetic Analyzer, Applied Biosystems). Similarity rank from the Ribosomal Database Project RDP) (Maidak et al., 1997) and FASTA Nucleotide Database Query were used to determine partial 16S rRNA sequences to estimate the degree of similarity to other 16S rRNA gene sequences. Analysis and phylogenetic affiliates of sequences was performed as previously described(Yakimov, 2006; Hassanshahian, 2010).

Results

Isolation and characterization of phenol utilizing bacteria

Eighteen phenol degrading bacteria obtained from marine environmental samples that collected from Persian Gulf. These different bacteria were subjected to the primary tests, i.e. Gram staining, oxidase, catalase, oxidative fermentative, motility, production of acid from glucose and growth under anaerobic and aerobic conditions, it was observed that all were Gram-negative rods, motile, oxidase and catalase positive, grew aerobically and were positive for production of acid from glucose. Results varied in the O/F test. Similar phenol degrading bacteria were eliminated and only 4 prevalent bacteria were selected for more study. (Singh et al.2013; Hassanshahian et al. 2012)

Growth rate and phenol removal by isolates

The results for phenol degradation and growth rate of all isolated bacteria were presented in table (1). As shown in this table all isolated bacteria have sufficient growth and phenol degradation, but four strains that showed high growth rate and phenol biodegradation (more than 70 percent) were selected for further characterization these selected strains were include : F6, F10, F13, F16. the degradation graphs for selected bacteria was shown in figure (1).

Emulsification activity (E24%) in isolated bacteria

The emulsification activity and bacterial adhesion to hydrocarbons were examined for all isolated bacteria for selection prevalent strains. The results for these two tests were shown in table (1). As shown in this table all isolated bacteria have well emulsification and hydrophobicity. The emulsification activity for selected strains was as follow: F6 (E24%71.68), F10 (E24% 74.67), F13 (E24% 75.83) and F16 (E24%73.78). Totally there was a direct relationship between emulsification activity and phenol biodegradation. As the degradative bacteria that have high emulsification they were better in phenol biodegradation.

Molecular identification of prevalent phenol-degrading bacterial strains

Molecular identification of the isolates was performed by amplifying and sequencing the 16S rRNA gene sequences and comparing them to the database of known 16S rRNA sequences. The results of the molecular identification showed that four isolated bacteria belong to these species F6: *Nitratireductor aquimarinus* Strain CL-SC21, F13: *Nitratireductor aquimarinus* Strain CL-SC22, F16: *Marinobacter hydrocarbonoclasticu*s Strain KMM3937 and F10: *Pseudomonas stutzeri* Strain GAPP4. The sequences from the isolated bacteria, entered with standard strain sequence in MEGA4 software. The phylogenetic tree was constructed using sequences of comparable regions of the 16S rRNA gene sequences that are available in public databases. The phylogenic trees of these four isolated strains were illustrated in Figure (2). Also the accession number for these four prevalent phenol degrading bacteria in the NCBI database was as follow: F6 (HG780369), F13 (HG780370), F16 (HG780362) and F10 (HG780372).

Discussion

In this study, 18 phenol-degrading bacterial strains isolated from seawater and sediment samples that collected from Persian Gulf. There are some reports from isolation and identification of phenol degrading bacteria from terrestrial and marine environments.

(Whitely, 2001; Srideri, 2012) collected 700 bacteria from an operational phenol remediation industrial treatment system, and found that *Pseudomonas* was the abundant taxon, and *P. pseudoalcaligenes* and *P. vesicularis* were the most common species.(Heinaru et al., 2000) found that out of 39 isolates, 21 were *P. fluorescens*, 12 were *P. mendocina*, four were *P. putida* one *P. corrugata*, and another was *Acinetobacter genospecies*.(Koutnay et al., 2003) isolated some phenol-degrading bacteria and also identified them as genus *Pseudomonas* sp.(PÃµllumaa et al., 2001)

While(Koutnay et al., 2003) showed that initial phenol concentrations up to 0.48 g phenol l^{-1} were completely degraded by *Pseudomonas* strains and some strains utilized phenol at up to 0.7 g l^{-1}, in the present study most strains could remove phenol up to 0.4 g l^{-1}. *P. stuzeri* strain GAPP4 had 79 percent phenol biodegradation.(Watanabe *et al*., 1999) also showed that there were changes in the major bacterial population in phenol digesting activated sludge in response to increases in the phenol loading rate.

The results of this study show that phenol degrading bacteria was important in Persian Gulf marine ecosystem and also these degradative bacteria have high diversity in the Persian Gulf. In this research two new genus that previously do not reported ans phenol biodegrader were reported these two new strains was: *Nitratireductor aquimarinus* Strain CL-SC21, F13: *Nitratireductor aquimarinus* Strain CL-SC22. By using of these phenol degrading bacteria in the biopiolt biodegradation system it is possible to decrease the phenolic pollution in the Persian Gulf.

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Table 1. Growth rate, emulsification activity and phenol biodegradation by isolatstrains

Phenol degradative isolated strains	Growth (O.D 600 nm)	Emulsification Activity (E24%)	Percentage of phenol degradation	BATH (%)
F1	0.732	70.56	71.36	60.11
F2	0.645	63.78	66.45	57.92
F3	0.794	71.45	70.53	59.36
F4	0.456	55.65	57.52	48.50
F5	0.645	67.89	61.45	55.81
F6	0.872	71.68	77.67	62.52
F7	0.756	72.57	72.56	60.78
F8	0.674	68.56	63.56	56.93
F9	0.345	59.67	50.54	47.24
F10	0.894	74.67	79.94	63.59
F11	0.495	63.67	52.78	49.58
F12	0.596	61.67	59.78	57.72
F13	0.856	75.83	75.89	61.69
F14	0.736	69.65	68.46	52.75
F15	0.728	71.75	70.52	57.09
F16	0.902	73.78	80.47	65.75
F17	0.701	70.46	69.45	57.82
F18	0.659	68.64	67.84	56.24

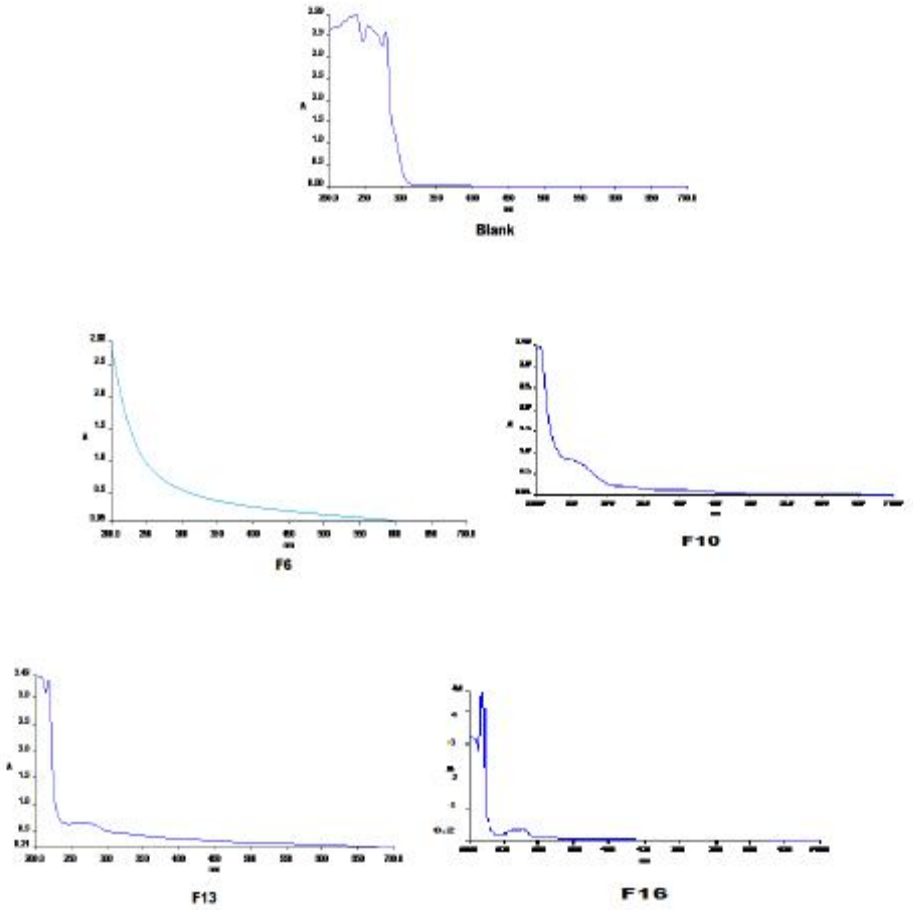

Figure 1. The absorbance graphs of phenol biodegradation by prevalent isolated strains

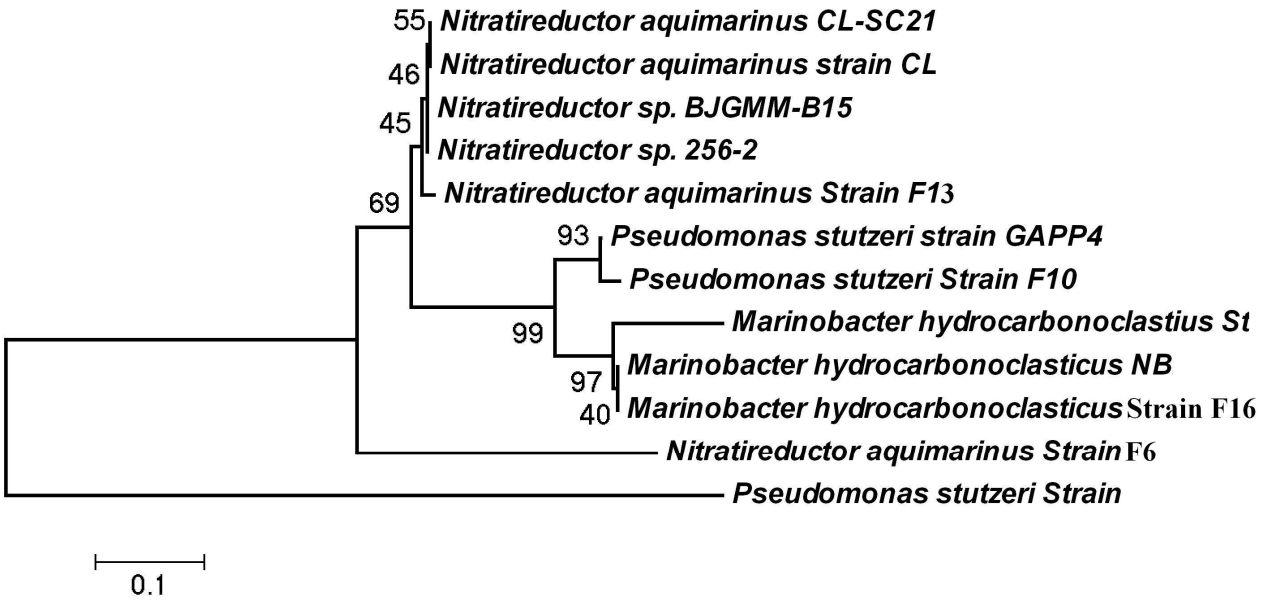

Figure 2. Phylogenetic tree based on partial 16S rRNA gene sequenced data showing the location of isolated strains. The scale represents 0.1 substitutions per base position.