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Original Research Paper

Optimization of phenol biodegradation by efficient bacteria isolated from petrochemical effluents

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ABSTRACT: Phenol is an environmental pollutant present in industrial wastewaters such as refineries, coal processing and petrochemicals products. In this study three phenol degrading bacteria from Arak Petrochemical Complex effluent were isolated which consume phenol. Molecular analysis was used to identify bacteria and isolated bacteria were identified as *Rhodococcus pyridinivorans* (NS1), *Advenella faeciporci* (NS2) and *Pseudomonas aeroginosa* (NS3). Among the isolated strains, NS1 had the highest ability to degrade phenol. In order to observe the best yield in phenol biodegradation using NS1, optimization was performed using one factor at a time of experimental design to investigate the effect of four factors, including pH, temperature, phosphate and urea concentration. The optimal biodegradation condition through or tho pathway was pH = 8, urea = 1 g/L, temperature = 30°C and K_2HPO_4 = 0.5 g/L. Under the suggested condition, a biodegradation efficiency of 100% was achieved. Moreover, NS1 has shown growth and phenol degradation in concentrations between 250 to 2000 mg/L. In a nutshell, the results revealed that phenol efficiently consumed by NS1 as the sole carbon source. Obviously, the isolate strain may be seen as an important tool in the bioremediation of wastewater effluent, petrochemical complex.

KEYWORDS: *Biodegradation; Effluents; Optimiztion; Petrochemical effluent; Phenol; Rhodococcus pyridinovorans*

INTRODUCTION

Many researchers investigate green and suitable technologies for the industrial wastewater treatments, especially in this era of industrial development (Kulkarni and Kaware, 2013). Although phenol is consumed in various industries as a crude material, but also it is considered as a toxic pollutant and the main organic constituents detect in petrochemical effluents, oil refineries, dye manufacturing, coke oven, fiberglass manufacturing, pulp and paper production, phenolic resin manufacturing, herbicide manufacturing, plastic and varnish industries (Paisio *et al*., 2013, Mohite *et al*., 2010, El-Ashtoukhy *et al*., 2013, Veeresh *et al*., 2005, Jadhav and Vanjara, 2004). World Health Organization (WHO) submitted 1 µg/L as a principle of phenol concentration in drinking water (Zhang *et al.,* 2009). Phenol is moderately soluble in water, generally substances which dissolve in water can be distributed in the water cycle faster and easier (Oller, *et al.,* 2011). Phenol is listed as priority pollutants by different agencies such as ATSDR and US EPA (Paisio *et al.,* 2012). Different procedures for phenol removal such as chemical oxidation, adsorption, liquid membrane permeation, coagulation, incineration and other nonbiological treatment procedure have disadvantages like their prices, manufacture some hazardous by-products and furthermore, some of these treatments

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use energy; ozone, radiation, etc. and chemical reagents; oxidizers and catalysts (Agarry *et al.,* 2008, Senthilvelan *et al.,* 2014, Muñoz *et al.,* 2005, Oller *et al.,* 2011). Aerobic biological degradation due to its complete mineralization and lower costs is generally preferred for effluents treatment. At high concentrations phenol is identified as an inhibitory material (Christen *et al.,* 2012). Although several species of bacteria are able to degrade phenol but among them *Rhodococcus* sp. due to its ability to degrade a wide range of complex synthetic molecules such as polychlorinated biphenyls, heterocyclic compounds and various herbicides is highly regarded. These substrates with their remarkable stability and toxicity cannot be easily degraded by other organisms (Warhurst and Fewson, 1994; Martínková *et al.,* 2009). Many elements such as acidity, different nutrients, temperature and incubation time can be influenced phenol degradation (Khleifat, 2006). Given that Arak Petrochemical Company is one of the largest petrochemical complexes in Iran, efficient treatment of wastewater can make a significant contribution to maintaining the health of the environment. Also, according to the fact that there is no comprehensive research on biodegradation optimization, this study can be useful and applied.

The present study was carried out to (1) isolate and characterize the phenol degrading bacteria from return activated sludge; (2) evaluate the isolated strains ability for phenol biodegradation and (3) and establish the effects of different conditions on the phenol degradation of *Rhodococcus pyridiniovorans* for the first time. This study has been performed in Shahid Beheshti University in Tehran, the capital city of Iran during 2013 to 2015.

MATERIALS AND METHODS

Sample collection

Samples were collected (n=3) from different parts (inlet and outlet of waste water treatment plant as well as activated sludge) of Arak Petrochemical Complex effluents randomly, kept at 4 ºC and transferred to the laboratory. Collected samples were used for the isolation of phenol degrading bacteria.

Isolation and identification of phenol-degrading bacteria

Some of sediment and collected water was added to mineral salt medium (MSM). Phenol in the culture medium was used as a carbon source (1000 mg/L). Incubation was carried out on orbital shakers. During of experimental period bacterial growth was monitored visually. After the growth occurred in the medium, about 5ml of the medium was incubated to a new conical flask containing 50 ml of MSM medium. This was repeated four times. Medium compositions was as follows: MSM medium (g/L): NH_4Cl, 1; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0/2; $FeSO_4.7H_2O$, 0/01; $CaCl_2$, 0/01 and 1 mL trace solution (Schlegel, 1992). In order to purification of bacteria in the enriched culture medium, serial dilution method was used. Different dilutions were spread on solid medium containing 1000 mg/L phenol. After purification of various bacteria, molecular method was used to identify bacteria. In current study two methods were used to extract genomic deoxyribonucleic acid (DNA). SET buffer was used for DNA extraction of NS1 (Sambrook *et al.,* 1989). NS1 molecular (16S rDNA) Identification was performed by polymerase chain reaction (PCR) using a pair of forward 27F and reverse 1492R primers. DNAs of two other strains (NS2 and NS3) were extracted by DNA extraction kit (Roche®-Germany). Amplification of the genes was conducted by PCR using the same forward primer and another reverse primer 1510 R. Primus 25 advanced® thermo cycler was used to perform PCR. The PCR conditions were as follow: Initial denaturation: 95°C, 5 min; denaturation: 35 Cycles at 94°C, 1min; annealing: 52 ºC, 1: 30 min; extension: 72 ºC, 1min and final extension: 72 ºC, 15 min. Amplification product sent to commercial provider for sequencing. Finally, using the BLAST comparisons were done between the obtained sequences with sequences in databases (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*).

Biodegradation of phenol by isolated strains

In order to recognize the ability of pure strains for biodegradation of phenol, MSM medium containing phenol with isolated strains (final optical density = OD_{600nm} 0.10) were incubated.

Biodegradation optimization

To observe the best yield in phenol biodegradation, optimization was performed using one factor at a time experimental design to investigate the effect of four factors, including pH (4-10), urea concentration (0.15 to 2 g/L), temperature (20-40 ºC), phosphate concentration (0.15 to 2 g/L). During optimization process phenol (1000 mg/L) was used and added to medium cultures. To achieve the initial number of

bacteria in culture media, firstly, NS1 was inoculated to the MSM medium (30 ºC and 48 hours). Secondly, bacteria were pelleted by centrifuge (15 min and 8000 rpm) and at the end bacteria washed and added to culture medium. Non-biological degradation of phenol in treatments was determined using the control treatments without inoculation. Treatments in orbital shakers (130 rpm) and OD = 0.10 with three replicates were done. In order to evaluate the accuracy of the optimization process, the optimized cultural conditions, were then applied to biodegradation of phenol.

Effects of different phenol concentration on biodegradation ability of NS1

At the end of establishing optimal degradation conditions, the NS1 was added to 50 ml MSM medium with different initial phenol concentrations (250, 500. 750, 1000, 1250, 1500, 1750, 2000 mg/L). Biodegradation ability of NS1 in different concentrations of phenol was determined every 12 hours. All flasks were inoculated with final optical density (OD_{600nm}) of 0.10 at 130 rpm with three replicate.

Phenol determination and cell growth

Concentrations of phenol in the samples were measured using colorimetric method. Phenols in the sample were coupled with 4- amino anti-pyrene and oxidation in alkaline conditions was performed by potassium ferricyanide. The red color that is produced in this reaction was measured at 500_{nm}. In order to determine the amount of remaining phenol in the samples standard curve was used (APHA, 1998). Bacterial growth was measured using optical density (600nm) against MSM as reference.

Statement of phenol degradation pathway

The ring fission process that causes 2 pathways for phenol biodegradation was exposed using the Rothera's reaction. After a sufficient cell growth on MSM agar plates augmented by 1mM phenol, it was added in Tris buffer 0.02 M, pH 8. One drop toluene and catechol (0.1 M, 0.2 ml) was added to the samples (2mL). After shaking at 30 ± 0.1ºC for 1 hour, by the modified Rothera's reaction existence of β-ketoadipate showed ortho cleavage (Kilby, 1947, Soudi and Kolahchi, 2011).

Statistical analysis

To compare the roles of temperature, pH, nitrogen and phosphate amounts on bacterial growth rates, One-way ANOVA was used. Significant differences were determined using Tukey's test. All analyzes were performed using SPSS 19 software.

RESULTS AND DISCUSSION

Identification of microbial strains

Due to extensive existence of phenol in the environment, a large number of microbial strains with ability to consume phenol have been identified (Basha *et al.,* 2010). Three morphologically different bacteria were found in the collected sample from Arak Petrochemical Complex effluent. Their identification were carried out by molecular analysis and results were deposited to Gen-bank (NS1: KP334125, NS2: KP150443, NS3: KP150444). It was indicated that strain NS1 had the highest similarity with *Rhodococcus pyridinivorans,* strain NS2 with *Advenella faeciporci* and strain NS3 with *Pseudomonas aeroginosa*. Among these three phenol degrading bacteria, strain NS1 with higher phenol degrading efficiency was characterized based on morphological, biochemical and physiological assays. On nutrient agar, NS1 colonies were 1–2 mm in diameter and beige. Strain NS1 was characterized as Gram positive coccobacilli. It was recognized to be a non-motile, non-spore-forming and colonies have irregularly round wrinkles in old cultures. The biochemical and physiological characteristics of the NS1 strain are presented in Table 1. *Pseudomonas* sp. whether pure or mixed is the most common microorganism for phenol biodegradation (Stoilova *et*

Table 1. Biochemical and physiological characteristics of the NS1 strain

Characteristics	NS1
Nitrates reduction	+
Indole production	-
D-glucose fermentation	-
H2S production	-
Oxidase	-
Catalase	+
Urease	+
Citrate	+
MRVP	-
Starch hydrolysis	-
Gelatin hydrolysis	-
Sugar assimilation	
Glucose	-
Saccharose	-
Maltose	-
Lactose	-
Mannose	-

Note: positive reaction = +; negative reaction = –

Table 2. Concentrations of residual phenol during 3 days experiment with the inoculation of isolated strains

Time (h)	NS1	NS2	NS3
24	970 (mg/L)	990 (mg/L)	980 (mg/L)
48	650 (mg/L)	740 (mg/L)	800 (mg/L)
72	120 (mg/L)	240 (mg/L)	500 (mg/L)

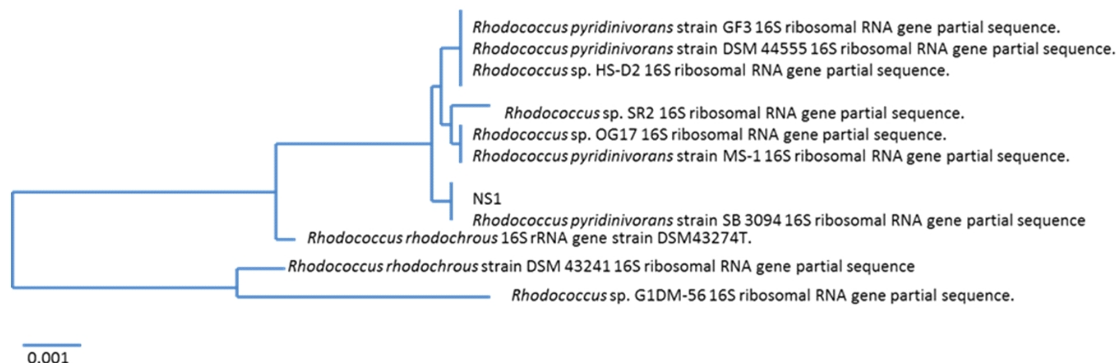

Fig. 1: Phylogenetic tree based on 16S rDNA gene sequence analysis showing the relationship between NS1strain and representative species of *Rhodococcus* genus

Table 3. Results of One-way ANOVA based on the optical density at the end of the 4-day experiment

Factors	F	p-value	Sum of Square
pH	16.12	0.00	0.334
Temperature	0.81	0.54	0.018
Urea (mg/L)	2.245	0.11	0.002
K_2HPO_4 (mg/L)	27.42	0.00	0.007

al., 2007) and they are believed to have good potential for different biotechnological applications (El-Naas *et al.,* 2010). So far, the ability of *Advenella* sp. to consume phenol is not mentioned in any documents. Because of the remarkable ability of *Rhodococcus* sp. to degrade various organic pollutants, it has attracted a lot of attention (Perry *et al.,* 2007). This genus plays a key role in nature and bioremediation conditions (Bell *et al.,* 1998). Given that *Rhodococcus* sp. is able to degrade various organic compounds, it highly regarded and several new species have been reported (Larkin *et al.,* 2005). According to the mentioned above NS1 was chosen for further studies (Table 2).

Phylogenetic analysis

The phylogenetic relationship among NS1 and *Rhodococcus sp*. illustrated in Fig. 1. Molecular analysis showed the NS1 has closest relation to *Rhodococcus* sp. Strain SB 3094. NS1 showed high homology with several *Rhodococcus* species (such as *R. pyridinivorans*, *R. rhodococcus*).

Factors affecting biodegradation of phenol by NS1

The results of One-way ANOVA based on the values of the optical density (OD_{600nm}) are reported in Table 3. The effect of three medium components and operating conditions were studied and among them pH and K_2HPO_4 (g/L) were found to be the significant variables ($p< 0.05$) for phenol biodegradation by NS1. Although in this study biodegradation accrued at the pH 7 to 9, but the pH 8 was chosen as the optimal condition (Fig. 2). Studies have shown each strain in certain pH shows the best biodegradation ability. For instance, the best condition for *Klebsiella oxytoca* was 6.8 and the best pH ranges for the biodegradation of phenol by *Halomonas campisalis* was determined between 8 and 11 (Khleifat, 2006). According to statistical analysis pH was found as effective factor in phenol degradation

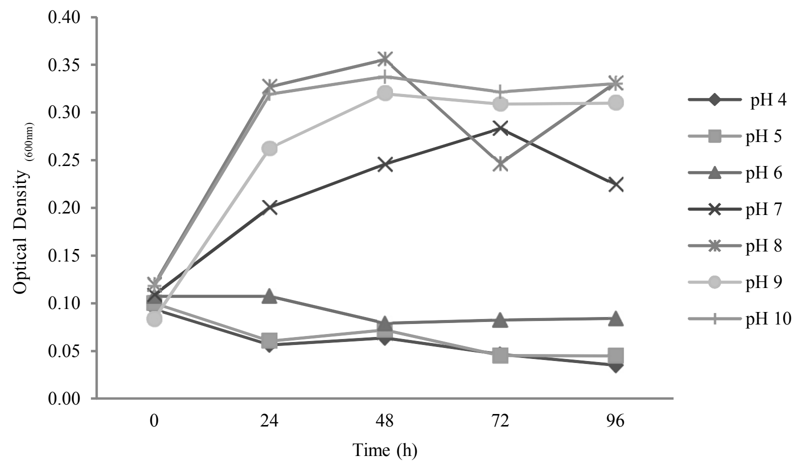

Fig. 2: Optical density of *Rhodococcus pyridinivorans* during the experiment under different pH

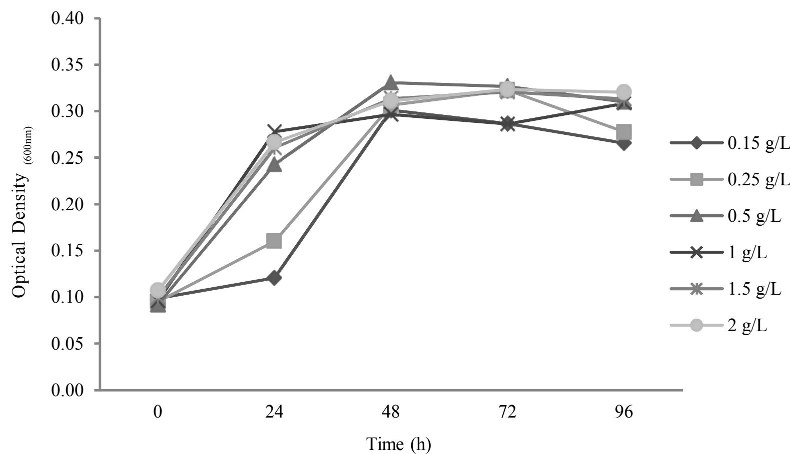

Fig. 3: Optical density of *Rhodococcus pyridinivorans* during the experiment under different phosphate concentrations

which may be because of its effects on stimulating the enzymatic activities, transportation and the nutrient solubility (Lin *et al.,* 2010). Most authors have shown that the supplemental nutrient source addition of nutrient accelerate the biodegradation of hydrocarbon pollution. Other studies have shown that fertilizer amendments produce no increase in biodegradation rates (Walworth *et al.,* 2007). K_2HPO_4 was another variable that showed significant effect on the phenol biodegradation in the current study (Fig. 3). Although urea showed no significant effects on phenol biodegradation at the select level (Fig. 4), indicating that biodegradation at low urea concentration is also possible. It has been argued that N concentration has ambiguous effects and excessive levels of N can result in deleterious/no effects (Zhou and Crawford, 1995). Temperature plays an important role in affecting petroleum hydrocarbons biodegradation, among the other environmental variables (Mohn annd Stewart, 2000). Moreover when the temperature increase, the bacterial metabolism increases as well (Leahy and Colwell 1990). Surprisingly, in this work, temperature did not show significant effect on phenol biodegradation (Fig. 5), according to the average temperature of the water in the area (≈30 °C) 30 ºC was considered as optimum level. After determining

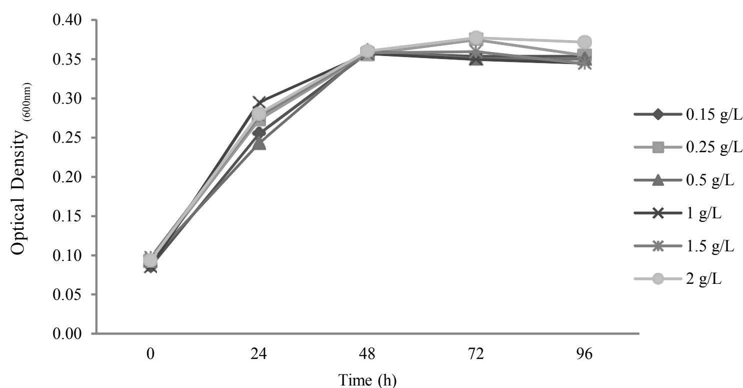

Fig. 4: Optical density of *Rhodococcus pyridinivorans* during the experiment under different urea concentrations

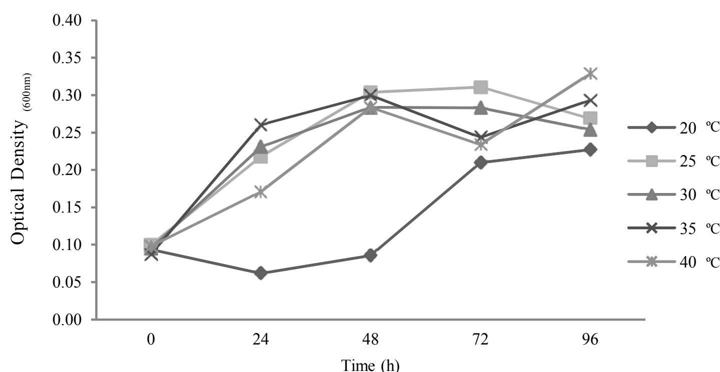

Fig. 5: Optical density of *Rhodococcus pyridinivorans* during the experiment under different temperatures

optimal biodegradation conditions by NS1, temperature = 30°C, pH 8, urea = 1 g/L and K_2HPO_4 = 0.5 g/L were considered as optimum conditions. Interestingly, adjusting the phenol degrading experiment with the above obtained optimized parameters resulted in the increasing of the phenol removal efficiency to 100 % in adjusted experiment in 2 days.

Effects of different phenol concentration on biodegradation ability of NS1

The results showed that, in all selected phenol concentrations (250 to 2000 mg/L) NS1 was able to degrade phenol (Fig. 6). The NSI degraded 250, 500 and 750 mg/L phenol completely in 24 hours. Research on the substrate concentration in phenol biodegradation is particularly important because phenol itself is an inhibitory material for microbial cells, especially at higher concentrations (Al-Khalid and El-Naas, 2012). Pradhan and Ingle (2007) showed that *Serratia plymuthica* strain GC had long lag phase when it was grown in the presence of high phenol concentrations (1050 mg/L). Similar events were shown by Wang *et al.,* (2007) who also remarked that phenol toxicity, caused the inhibitory effects on microbial growth at high concentrations of phenol, the results of the present study also confirm the mentioned studies above. Based on findings, it is clear that phenol consumption is strongly dependent on the initial phenol concentration due to toxic effects induced by the substrate.

Enzyme assay

Definite characteristic enzymes such as C23O (2,3 catechol dioxygenase) and C12O (1,2 catechol dioxygenase) were studied to distinguish between two different catechol degradation pathways (known as meta and ortho). Khleifat (2006) reported that the meta-pathway is the most useful form of catechol

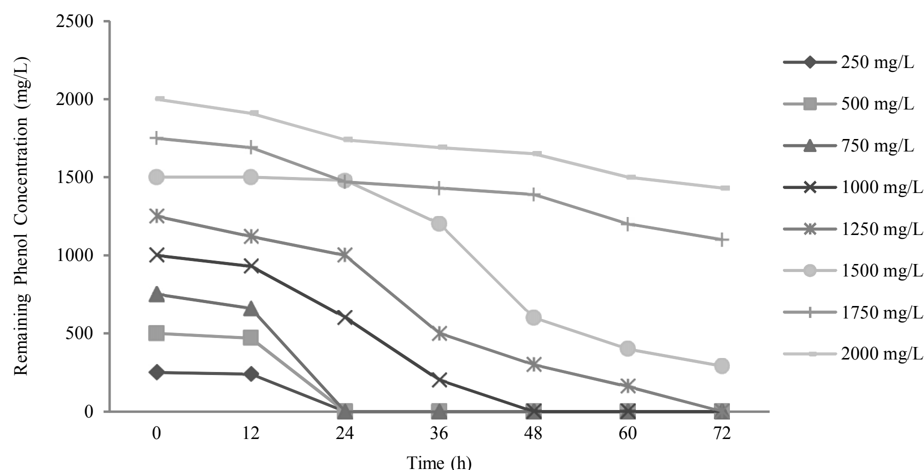

Fig. 6: Remaining phenol concentrations in the medium under different phenol concentrations

degradation for microorganisms such as *Ewingella americana* and Similar results were shown for strains of *Bacillus cereus* (Banerjee and Ghoshal, 2010). Ortho degradation pathway is reported in Pseudomonas sp., *Acientobacter calcoceticus* and *Candida tropicalis* (Basha *et al*., 2010). In this study also results showed that NS1 degrade phenol through the ortho pathway.

CONCLUSION

In a nutshell, a *Rhodococcus pyridinivorans* Strain named NS1 was isolated and characterized, from return activated sludge of the Arak petrochemical effluent, after enrichment on phenol. The results revealed the significant ability of NS1 to consume phenol. Obviously, NS1 is able to be seen as an important tool on bioremediation of wastewater effluent, petrochemical complex.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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