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ORIGINAL ARTICLE

Removal of phenolic compounds from Spent caustic wastewater by the isolated and detected *Pseudomonas otitidis*

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ABSTRACT
Spent caustic is one of the phenolic industrial pollutants produced by the chemical processes in petrochemical plants and oil refineries with high degrees of Chemical Oxygen Demand (COD), pH, salinity and sulfur compounds. The main goal of this study was isolation, identification and characterization of an efficient phenol removing bacterium from spent caustic waste water from Isfahan refinery, Iran. Enrichment of the samples was performed in basal mineral medium containing phenol as the sole carbon and energy sources and R_2A agar medium. For assessment of the ability of bacteria for phenol removal, the phenol broth media with 0.2 g L^{-1} phenol; and for determining the maximum inhibitory concentration, the phenol broth media with different phenol concentrations were used. Gibbs method was used for assessing phenol removal. The growth condition was optimized by Taguchi method. The biochemical and molecular analysis indicated the best phenol removing isolate resembled, Pseudomonas otitidis by 99% identity. Pseudomonas otitidis was able to grow in alkaline conditions with the pH value up to 10.5 and phenol concentration of 0.9 g L^{-1}. The optimum temperature for the isolate was 30°C. The isolate was able to reduce COD from 23100 mg L^{-1} to 1000 mg L^{-1}.
KEYWORDS *Spent caustic waste water, chemical oxygen demand, phenol removal, Pseudomonas otitidis, Taguchi method*

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INTRODUCTION

Phenolic wastes are of the most toxic pollutants for environment. Spent caustic is a phenolic chemical waste has not been easily degraded. There are many physical and chemical methods for treatment but these methods are expensive and produce secondary products .In the recent years biological methods are interested for treatment of spent caustic wastewater [1]. Since the dawn of petroleum refining, caustic solutions containing sodium hydroxide (NaOH) which has been consumed to remove acidic compounds [2,3]. Spent caustics usually contain pH higher than 12, sulfide concentrations beyond 2-3% wt. and salinity of about 5-12 % wt.[4]. Spent caustics contain high concentration of hydrogen sulfide with high chemical oxygen demand, and a variety of organic sulfur compounds and aromatic hydrocarbon compounds which are commonly named phenols, benzene and toluene [3,5]. Due to environmental regulations, and treatment costs of spent caustics are high. The purpose of recycling of this wastewater is to reduce the mentioned costs [3]. One of the most commonly adopted physical methods is Wet Air Oxidation (WAO) treatment which is liquid-phase hydrothermal oxidation consuming dissolved oxygen at

elevated temperatures, through air and supplementary steam injection. In this process, soluble or non-soluble compounds are partially or completely oxidized at elevated temperatures and pressures consuming air-oxygen as the oxidizing agent. WAO method oxidizes in order to reduce the sulfides and mercaptans. In chemical oxidation, hydrogen peroxide (H_2O_2) is widely consumed oxidant, which most often converts dissolved sulfide to thiosulfate and hence remains as chemical oxygen demand in the treated water by its incomplete oxidation. A new method for biological treatment of spent caustic is introduced. In biological treatment spent caustic waste water is degraded at atmospheric pressures and temperatures. Biological treatment would be a cheaper and safer alternative in comparison with the available physicochemical treatments [3, 6]. The aim of this study was isolation, identification and characterization of an efficient phenol removing bacterium from spent caustic waste water.

MATERIALS AND METHODS

Sampling and Chemical Testing

For screening and isolation of phenol removing bacteria, spent caustic wastewater was collected from Isfahan petroleum refinery, Iran in Jan 2014 and transferred to the microbiology laboratory of Isfahan University in sterile conditions and stored at 4°C until use [7]. For determination of the spent caustic wastewater characteristics, NaCl, pH, and total dissolve solids (TDS) were measured by Mettler Toledo multi meter device [7]. The rate of reduction and chemical oxygen demand (COD) was measured by distillation return package, and the rate of biochemical oxygen demand (BOD) was measured by titration method [8].

Media, Enrichment and Bacterial Isolation

A phenol broth medium containing (gram per liter of distilled water): KH_2PO_4, 2.25; $(NH_4)_2SO_4$, 1.0; $MgCl_2.6H_2O$, 0.2; NaCl, 4; $FeCl_3.6H_2O$, 0.02; $CaCl_2$, 0.01 and phenol (200 mgl^{-1}) as the only carbon source was used for isolation of phenol degrading bacteria. The pH value was adjusted to 7-7.2 [9]. After inoculation of the samples, the media were incubated on shaker (150 rpm), at 30°C. The sample enrichment lasted one month, during which, each week 1 ml of the media was inoculated to 100 ml of new phenol broth medium. After the last passage, serial dilution of cultured sample up to 10^{-7} was prepared and 0.1 ml from 10^{-5}, 10^{-6} and 10^{-7} dilutions were placed on R_2A agar media. The plates were incubated at 25°C for the purpose of bacterial isolation [10].

Selection of Phenol Degradation Bacteria

For assessing the ability of bacteria for phenol removal, the phenol broth media with 0.2 g L^{-1} phenol concentration were used. Bacteria were incubated in flasks on shaker at 150 rpm, at 30°C for one week, and finally absorbance was read and recorded in the wave length of 600 nm [10, 11].

Determination of Maximum Inhibitory Concentration of Phenol

Twenty ml of phenol broth media were added with different concentrations of 0.2-0.9 g L^{-1} phenol in separated Erlenmeyer flasks, and then 5 ml of media including 5×10^8 bacterial cell were added to each tube. These media were incubated for 24 hours in 25°C and then, their absorbance was read at 600 nm [12].

Determination of Phenol Removal

For assessing phenol removal by the isolated bacteria, Gibbs method was used. In this method 2-4 dichloroquinone-4-chloromide (Gibbs indicator) used which reacts to phenol and creates blue color complex. After centrifugation of the phenol broth media (in 6000 rpm for 10 min), 150 µl of media supernatant (pH value was adjusted to 8) were mixed with 30 µl of $NaHCO_3$ (pH value was adjusted to 8) followed by adding 20 µl of Gibes indicator (1 mg L^{-1}) and mixed by vortex. The mixture was placed for 15-45 min at room temperature. Finally, the mixture absorbance was read and recorded at 630 nm [13].

Growth of the Best Isolate in Spent Caustic Waste water

The most important indicators for best strain activity were high phenol, pH and temperature tolerance. The growth condition of these bacteria was optimized through L 16 array Taguchi experiment design (Qualitek-4 software) in a phenol broth media with addition of 100 ml spent caustic [14, 1]. After the growth of bacteria, the rates of COD reduction in the media were measured.

Identification of the Microorganism

Identification of the isolated bacterium was conducted based on colony morphology, biochemical testing, Gram staining, motility, SEM electron microscopy (XL30 ESEM) and molecular analysis [15]. DNA was extracted from cultured bacterium by simple boiling method. In this method the colonies or pellets were suspended in 500 µl of distilled water and heated for 10 minutes in boiling water bath. The layside cells were centrifuged in 12000 rpm for 10 minutes and the extracted cells were washed by distilled water three times. Amplification reaction was done in 50 µl reaction volume using universal primers (RW01: 5′-

AAC TGG AGG AAG GTG GGG AT-3′; and DG74: 5′-AGG AGG TGA TCC AAC CGC A-3′). The reaction tubes contained 0.3 µM of each primer, 0.2 mM dNTPs, 1X PCR buffer, 1.5 mM $MgCl_2$, 1U Taq DNA polymerase, 1 µg template DNA extracted from the bacterium and DEPEC water up to 50 µl. The PCR reaction took place in an Eppendorf thermal cycler by appropriate program optimized for the primer pairs. The PCR program included 35 cycles and each cycle consisted of the following stages: denaturation, at 94°C for 2 min; primer annealing, at 55°C for 1 min and extension, at 72°C for 1minute. The PCR product was detected by 1% agarose gel electrophoresis in 1X TBE buffer. The band was visualized on UV Transilluminator (Emtiazi et al., 2013). The partial 16S rRNA gene sequence containing 370 nucleotides was amplified. For sequencing, the product was sent to Gen Fan Avaran Company, Tehran, Iran. The sequences were corrected by Finch TV V.1.4.0., and were aligned in Gene bank using Mega 4 software

RESULTS AND DISCUSSION

Chemical Testing

Physical properties of spent caustic wastewater were measured and results shown in table 1 indicate that spent caustic wastewater is alkaline with high salinity, COD and TDS.

TABLE 1. Chemical Properties of the spent caustic Waste Water Sample.

pH	NaCl (mg L^{-1})	COD (mg L^{-1})	BOD_{5days} (mg L^{-1})	TDS (mg L^{-1})
12.67	11512.8	23100	370	79750

Removal of Phenol by Isolated Bacteria

During one month of enrichment 4 isolates obtained which were able to grow in phenol broth media with 0.2 g L^{-1} phenol. The results of growth rates are shown in Figure 1.

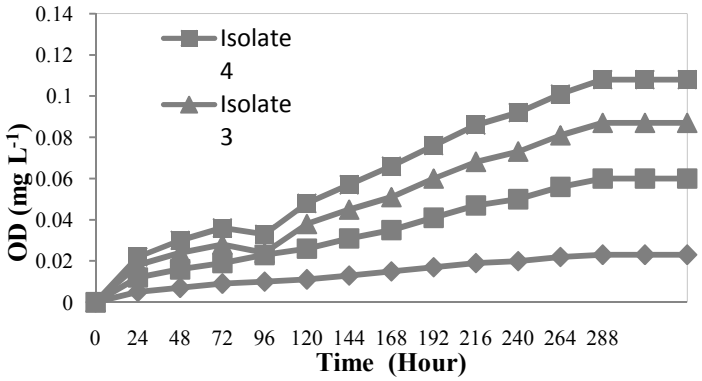

Fig. 1. The growth curve of 4 isolates which were able to grow in phenol broth media with 0.2 g L^{-1} phenol. The most growth ability belongs to the isolate 4.

Maximum Inhibitory Concentration of Phenol on Selected Isolate

Among the isolated bacteria, one (isolate 4) selected which was able to grow best in the presence of phenol. As shown in Figure 2, the bacterium is able to grow in the media containing up to concentration of 0.9 g L^{-1}phenol.

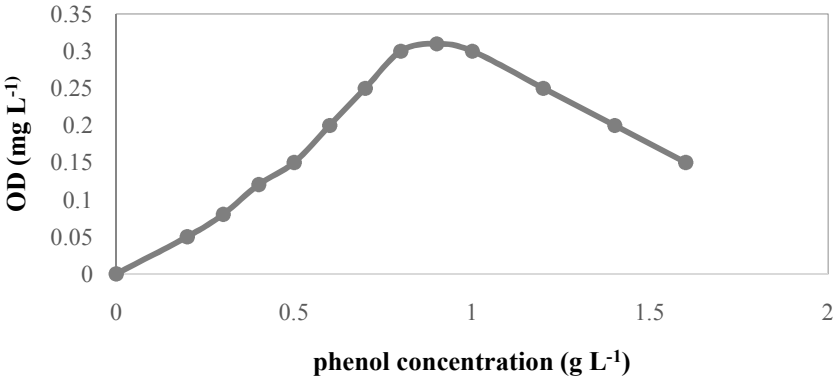

Fig. 2. Growth curve of the best isolate (4). This bacterium shows most OD in 0.9 g L^{-1} of phenol concentration.

Phenol Removal by Selected Isolate

Through Gibbs method was determined that the best isolate is able to remove 0.9 g L^{-1} phenol during 48 hours in broth medium.

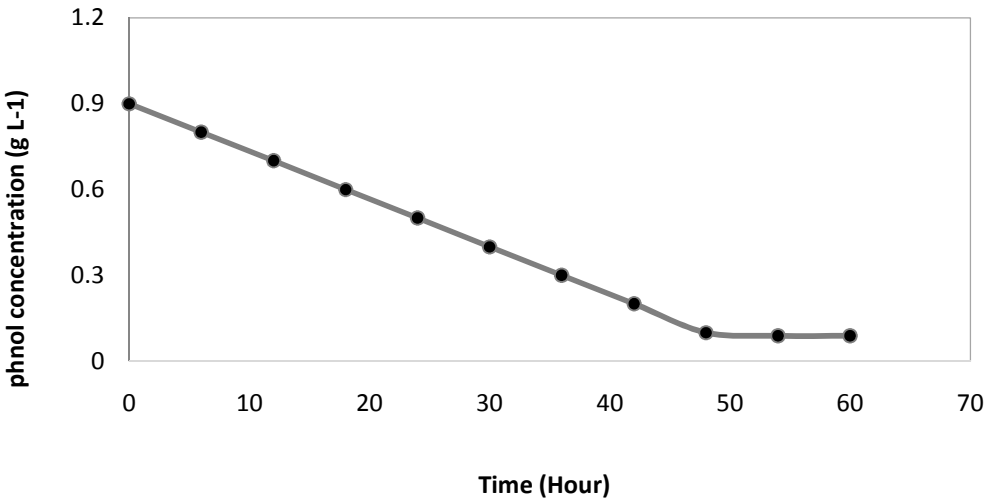

Fig. 3. Elimination curve of phenol for best strain with Gibbs method for 48 h.

Identification of the Isolate

General identification tests indicated that the isolate 4 is a motile Gram-negative rod with the biochemical properties which shown in Table 2. According to obtained results *Pseudomonas* sp. was initially determined.

TABLE 2. The Results of Biochemical Tests for Initial Identification of the Selected Isolate.

Oxidase	Catalase	BA	Lactose fermentation	Glucose fermentation	Gas from glucose	Endole production	VP	MR	Urea hydrolysis	Citrate utilization
+	+	β hemolysis	-	-	-	-	-	-	+	+

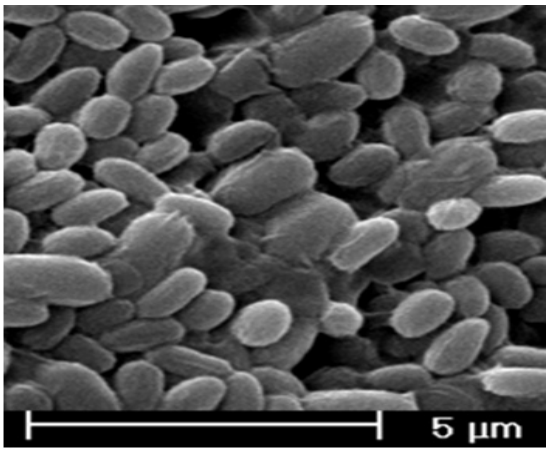

Fig. 4. SEM electron micrograph shows the morphology of cells after exposure to phenol. Flagellated rods with almost wavy surfaces resemble to Gram-negative bacteria are illustrated.

Molecular Analysis

The amplified PCR product was approximately 370 bp in length (Figure 5). Alignment of the sequence indicated that this strain resembles *Pseudomonas otitidis* with 99% similarity.

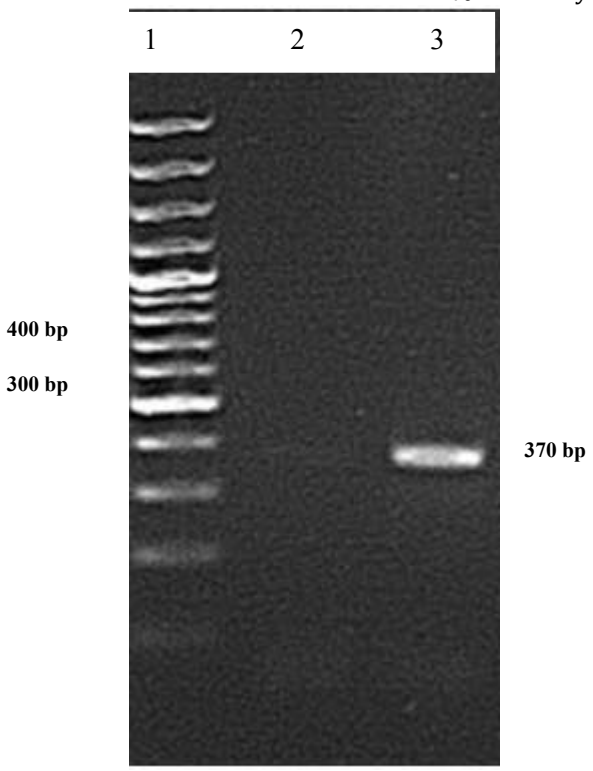

Fig. 5. The PCR product detected from amplification of 370 bp fragment in 16S rRNA gene (lane 1: 100 bp DNA ladder; lane 2: negative control; lane 3: 370 bp band from amplification of template DNA).

The Best Growth Conditions in Spent caustic Waste Water

The best grow temperature for the isolate 4 was 30˚C. The isolate was able to grow in alkaline conditions with pH value up to 10.5 and optimized phenol concentration up to 0.9 g L^{-1}. In mentioned optimized condition the isolate was able to reduce COD from 23100 mg L^{-1} to 1000 mg L^{-1}.

In the recent years due to increasing industrial activities and high amounts of petrochemical pollutions production, industrial and semi-industrial societies are faces to environmental problems [16]. Petroleum waste waters are refined through the common physical and chemical procedures, but the inefficiency and high cost of the procedures have made them to be replaced by new procedures such as biological methods. One of the most effective bacteria in biodegradation processes is the members of *Pseudomonas* sp. which are capable for degradation of aromatics [1].

In the present study during one month of enrichment 4 strains were isolated from spent caustic waste water which were able to grow in phenol broth media with 0.2 g L^{-1} phenol and among these 4, one strain with the best growth was selected. The best isolate was identified as *Pseudomonas otitidis* based on biochemical tests and molecular analysis. The bacterium was able to grow in 0.9 g L^{-1} phenol. Hassanshahian *et al.* [17] isolated and identified phenol degrading bacteria from polluted soil in Isfahan, Iran. All identified isolates consumed 0.2 g L^{-1} phenol and were identified as *Pseudomonas* spp. Kafilzadeh *et al*. [18] assessed the biodegradation of phenol in Parishan Lake, Fars, Iran and found that, *Pseudomonas*, *Acinetobacter, klebsiella, Citrobacter, Shigella* are predominate bacteria respectively.

Different methods have been used through the investigators to analyze phenol removal. Wantanabe *et al*. [19, 20] used Calori-metric method for determining the remaining phenol in media [19, 20]. Abavisani *et al*. [1] analyzed phenol with high performance liquid chromatography (Abavisani et al., 2015).

In this study, through Gibbs method and applying 2, 4 dichlo-roquinone-4-chloromide indicator, the phenol concentration was measured. The result of phenol analyze, using this method, is similar with results of other researchers [17].

Also Abavisani *et al.* [1] isolated *Halomonas*, *Janibacter* and *Pseudomonas* from spent caustic waste water in Iran, which were able to biodegrade phenol. The strain *Janibactersp.*YF3 was selected because of its high phenol-removal rate. The strain was gram positive coccus and optimal growth condition was 30 ºC,

Nacl 5 % (w/v), pH 8.5 and concentration of phenol 400 mg L^{-1}, but this study showed that best bacterium in spent caustic waste water from Isfahan refinery was *Pseudomonas otitidis*. The isolate was able to grow in alkaline conditions with pH value up to 10.5 and optimized phenol concentration up to 0.9 g L^{-1} [1].

CONCLUSION

In this study it was found that *Pseudomonas* sp. were predominate in spent caustic waste water and were powerful for bioremediation of phenol. The maximum grow temperature of isolated bacterium was around 35ºC. However it has been shown that phenol hydroxylase which is the key enzyme for phenol degradation pathways is usually sensitive to temperatures higher than 35ºC and significantly loses its activity (Al-Khalid and El- Naas, 2012). But unexpectedly, the isolated *Pseudomonas otitidis* was able to grow at temperatures around 35ºC with the best growth at 30ºC. In this study despite the extreme condition of the experiment, *Pseudomonas otitidis* was able to remove phenol up to 0.9 g L^{-1}. Due to the amount of phenol in wastewater which was less than this concentration, the isolate can be sufficiently used for treatment spent caustic or other phenolic wastewaters.

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