# Isolation and Genetic Identification of Phenol Degrading Bacterium from Wastewater of Assiut University Hospitals

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## Abstract

Phenol and phenolic compounds are the most common pollutants in hospital wastewater. Their carcinogenic and toxic effects have been recorded on human being. Identification of the key microorganisms that play a role in pollutant degradation processes is relevant to the development of optimal in situ bioremediation strategies. In the current study, a bacterium strain designated as AUN-AS01 was isolated from wastewater of Assiut University hospitals by enrichment technique in mineral basal salts (MBS) medium supplemented with phenol as a sole carbon and energy source. The strain AUN-AS01 was identified using PCR amplification of 16S rRNA gene and sequence analysis. The comparison of the alignment results and phylogenetic analysis of the sequences of the isolated strain to published rRNA gene sequences in Gen Bank, confirmed the identification of the isolate as *Paenibacillus mucilaginosus* AUN-AS01.The strain was able to grow and had a tolerance of phenol concentration up to 1600 mg/$L^{-1}$. It was observed that temperature, pH and initial concentration of phenol play key roles in determining the rate of phenol degradation by the isolated strain AUN-AS01. Results showed that, the strain was efficient in removing 92.26±0.05% of the initial 800 mg/l phenol within 48 h with optimal conditions, at 30 $^{o}$C and had a pH of 7.0. Our results demonstrate that, strain *Paenibacillus mucilaginosus* AUN-AS01could be used to remove the phenol from the environment. These findings may lead to new

biotechnological applications for the degradation of phenol, related to hospitals wastewater.

***Keywords: Bacteria; Isolation; Biodegradation; Phenol pollutant; 16S rRNA gene sequencing; Phylogenetic analysis.***

## 1. Introduction

Phenol and phenolic compounds are the most common pollutants in hospital wastewater (Arutchelvan, et al., 2007; Mohanty et al., 2017). They have been classified as a highly hazardous chemical (Hooived et al., 1998), and have been included in the list of priority pollutants by the U.S. Environmental Protection Agency (EPA, 1977). It is very important to remove phenol from contaminated water before discharge into any natural water because of their toxicity to aquatic organisms (Sachan et al., 2019). It has severe effect on human being, both short and long term (Sonawane and Koreke 2016).

Phenol removal by biological methods using microorganisms is preferred to physicochemical methods because of its eco-friendliness and cost effective nature and the possibility of complete mineralization of the phenol substrate (Kobayashi and Rittmann, 1982; Thakur, 2004; Prpich and Daugulis, 2005).

Microorganisms produce some enzymes that are effective in treating the industrial effluents containing phenol and its derivatives (Dubey and Hussain 2014). They can utilize phenol as the sole source of carbon and energy (Barbosa et al., 1996). Different microorganisms belonging to bacteria, yeasts, algae and fungi have been reported for their ability to degrade phenol and its derivatives (Sivasubramanian and Namasivayam, 2014).

Therefore, the objectives of this study were: (i) to isolate phenol-degrading bacteria from hospital wastewater, Assiut University; (ii) to identify phenol-degrading bacteria at the species level using polymerase chain reaction (PCR)-based amplification and sequence analysis of the 16S rRNA gene; and (iii) to optimize different parameters to enhance the rate of phenol degradation by the isolated bacteria.

## 2. Materials and Methods

### 2.1. Wastewater Sample, isolation and selection of phenol degrading bacteria by enrichment technique:

For isolation of phenol-degrading bacteria,(Explain: no of samples & collection method) the sewage wastewater samples were collected from Assiut hospitals area of Assiut University, Assiut. About 5 ml of each sample was suspended in 100 ml of minimal salt medium (MSM) containing $KH_2PO_4$ 0.5 g, $K_2HPO_4$ 0.5 g, $CaCl_2$ 0.1 g, NaCl 0.2 g, $MgSO_4.7H_2O$ 0.5 g, $MnSO_4.7H_2O$ 0.01 g, $FeSO_4.7H_2O$ 0.01 g, $NH_4NO_3$ 1.0 g per liter double distilled water. 10 mg/l of phenol was used as sole source of carbon and then incubated in 250ml flask at $30^0$C on rotary shaking incubator at 120 rpm for a week (Nagamani *et al*, 2009). A volume of 5 ml of enriched medium was transferred into freshly prepared media on each week supplemented with 10mg/, and then incubated at $30^0$C. The final enriched medium were spread on MSM agar plates supplemented with phenol (500 mg/l). The plates were incubated at 30 $^o$C and single colonies with morphological differences were selected and they were picked for purification. Pure bacterial isolates were obtained by repetitive streaking on MSM-phenol agar-coated plates and kept on the same MSM slant agar culture at 4◦C.

### 2.2. Molecular Identification:

#### *2.2.1.* DNA Isolation and PCR Amplification of 16S rRNA Gene:

The genomic DNA from the isolated bacterium was extracted according to the method described by Hesham (2014) and amplified by PCR using 16S rRNA universal primers 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-CGGCTACCTTGTTACGACTT-3) (Lane, 1991). A PCR was performed in 50µl as a final volume containing GoTaq (Promega, Madison, WI, USA) green master mix, 1µl DNA sample and each primer 1µl (at a concentration of 0.5 mM). PCR was performed with the following program: 5-min denaturation at 95 ◦C, followed by 36

cycles of 1-min denaturation at 94 ◦C, 1-min annealing at 55 ◦C, 1.5-min extension at 72 ◦C and a final extension step of 7 min at 72 ◦C. Five µl of the amplified mixture was then analyzed using 1.5% 0.5 × TBE agarose gel electrophoresis. The gel was stained with ethidium bromide, visualized under UV light and photographed.

### *2.2.2.* PCR Products Purification and Sequence Determination:

To verify the presence of appropriate sized amplicons, the PCR product for The selected was subjected to electrophoresis in 1% agarose gel according to standard methods. Product of the correct size was purified with a TaKaRa Agarose Gel DNA Purification Kit version 2.0 and sequenced in both directions using an ABI 3730 automated sequencer (Macrogen, Seoul, Korea).

### *2.2.3.* Comparison of 16S rRNA Gene Sequences with GenBank Database:

The 16S rRNA gene sequences of the selected isolate obtained in this study were aligned and compared with the known 16S rRNA gene sequences in the GenBank database using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (http://www. ncbi.nlm.nih.gov/BLAST/) to determine the closest available database sequences.

### *2.2.4.* Phylogenetic Analysis:

To determine the taxonomic position of the isolate, a phylogenetic tree was constructed with MEGA version 4.0 using a neighbor-joining algorithm, and the Jukes–Cantor distance estimation method with bootstrap analyses for 1000 replicates was performed (Hesham et al., 2016).

## 2.3. Phenol Degradation:

The culture of strain AUN-AS01 was prepared and adjusted to an optical density at 600 nm (OD600) of 1.0, then the final concentration of 2% (v/v) inoculums were inoculated into the flasks containing MSM medium with phenol as sole carbon source. The

range of phenol concentrations was increased from 100 to 2500 mg/l. The flasks were incubated at 30 $^0$C with 120 rpm for 2 days. Samples were collected to measure the bacterial biomass and the phenol degradation. The biomass contents were monitored spectrophotometrically by measuring absorbance at 600 nm. The phenol concentrations were determined by using 4-aminoantipyrine in the colorimetric assay, according to standard methods reported by the American Public Health Association (APHA, 2005). All experiments were conducted in triplicate.

### 2.4. Optimization of culture conditions for phenol biodegradation:

Factors affecting phenol degradation by the selected bacteria, including pH (5–11), temperature (20–45°C) and initial phenol concentration (100 mg/L to 2500 mg/L) were investigated. All experiments were conducted in triplicate.

## 3. Results and Discussion

### 3.1. Isolation and characterization of phenol tolerating bacteria:

Several bacterial isolates were isolated from the enrichment cultures of the hospital wastewater samples. Based on its ability to survive the high concentration of phenol , the isolate designated as AUN-AS01was selected for further study. The selected isolate was Gram negative and rod-shaped cells.

### 3.2. Identification Using 16S rRNA Gene Sequencing and Phylogenetic Analysis:

In order to identify and determine the correct phylogenetic position of the selected isolate, molecular genetics identification was performed. In this way, the genomic DNA was extracted from the selected bacterium AUN-AS01 and universal primers 27F and 1492R were used for the amplification and sequencing of the 16S rRNA gene fragment. Alignment of 16S rRNA gene sequences of the selected bacterium AUN-AS01 with published 16S rRNA

sequences from Gen Bank using BLAST shows identity 100% with *Paenibacillus mucilaginous*.

### 3.2.1 Phylogenetic Tree Analysis:

Phylogentic tree was constructed for AUN-AS01 strain along with other sequences of the same genus from GenBank to confirm the correct position of the strain. As shown in Fig. 1, strain AUN-AS01 and *Paenibacillus mucilaginosus* share one clade. Therefore, strain AUN-AS01 was identified as *Paenibacillus mucilaginosus.*

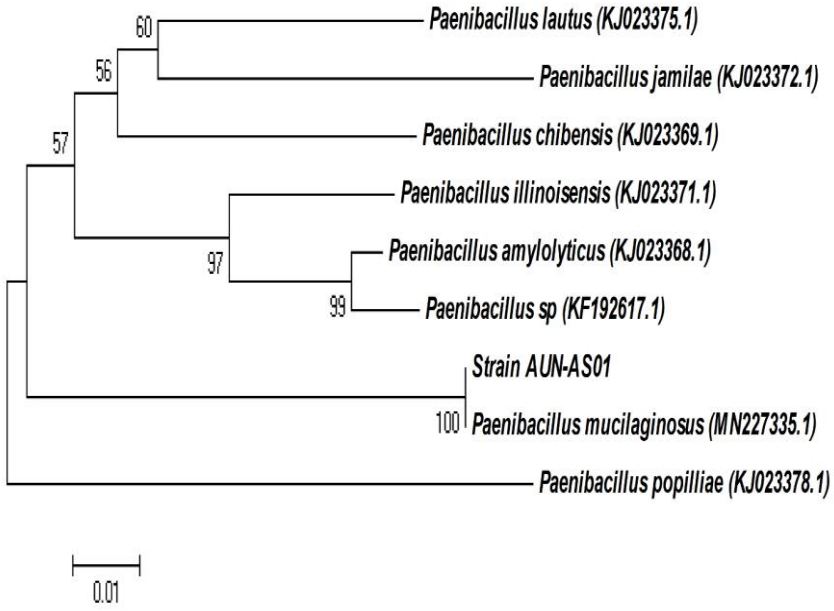


**Fig. 1: Phylogenetic tree relationships between strain AUN-AS01 and 16S rDNA sequences from other published *Paenibacillus* spp. GenBank accession numbers are given in parentheses**

Gene comparison studies have shown that 16S rRNA is highly conserved within a species and among species of the same genus (Abd-El-Haleem, et.al., 2002)., identification based on 16S rRNA gene sequencing and phylogenetic analysis was used for the

bacterial species (Franzetti., 2007; Zhang et al., 2012; Panda et al., 2013; Hesham et al., 2014a, b and Hesham et al., 2016).

### 3.3. Phenol Biodegradation by *Paenibacillus mucilaginosus* AUN-AS01:

The phenol-degradation rates and biomass of *P. mucilaginosus* AUN-AS01 at various initial concentrations of phenol (200–2500 mg/ $L^{-1}$) were determined by monitoring phenol concentration and cell growth at OD600 at the end of experiment . The degradation of phenol and the biomass growth of *P. mucilaginosus* AUN-AS01 were increased by increasing the phenol initial concentrations. The maximum biomass and the rate of phenol degradation were noticed at the initial phenol concentration of 800 mg/ $L^{-1}$ with the degradation rate of 92.26±0.05 % (Table 1). Results in Table 1 also showed that an inhibitory effect was occurred in the biomass growth and the degradation rate of phenol with the elevated initial phenol concentration higher than 800 mg/ $L^{-1}$, whereas there was no growth of phenol-degrading bacterium when the initial phenol concentration was higher than 1600 mg/ $L^{-1}$.

Many studies on biodegradation of phenol using pure and mixed bacterial strains capable for phenol degradation have been reported by various authors, (Vione et al. 2005; Geng et al. 2006; Stoilova et al. 2006; Chandra et al., 2011; Renard et al., 2016; Suheir et al., 2018). In addition to, Chakraborty et al, (2010) investigated the biodegradation of phenol by native bacteria strains isolated from coke oven processing wastewater. Chandra et al, (2011) reported that phenol is not easily biodegradable and inhibits the innate activity of most of the microbes at higher as well as lower concentrations. This observation is well correlated with our findings.

**Table 1. Biomass of bacterial cell growth (*Paenibacillus mucilaginosus* AUN-AS01) at OD600 and rate of phenol degradation at various initial concentrations of phenol from 200–2500 mg/ $L^{-1}$.**

| Initial phenol concentrations (200–2500 mg/ $l^{-1}$) | *Paenibacillus mucilaginosus* AUN-AS01 | |
|---|---|---|
| | % Phenol removal | Growth rate (Biomass) $OD_{600}$ |
| 100 | 7.31±0.09 | 0.24±0.01 |
| 200 | 9.11±0.06 | 0.37±0.03 |
| 300 | 12.23±0.06 | 0.47±0.03 |
| 400 | 18.78±0.07 | 0.54±0.06 |
| 500 | 24.26±0.06 | 0.63±0.03 |
| 600 | 44.18±0.06 | 0.70±0.07 |
| 700 | 68.20±0.06 | 0.84±0.04 |
| 800 | 92.26±0.05 | 1.04±0.03 |
| 900 | 79.32±0.05 | 0.91±0.02 |
| 1000 | 70.23±0.06 | 0.79±0.01 |
| 1100 | 60.16±0.06 | 0.69±0.01 |
| 1200 | 53.71±0.06 | 0.62±0.02 |
| 1300 | 41.53±0.04 | 0.55±0.03 |
| 1400 | 32.35±0.06 | 0.49±0.02 |
| 1500 | 22.19±0.06 | 0.42±0.02 |
| 1600 | 17.69±0.06 | 0.34±0.02 |
| 1700 | 11.10±0.06 | 0.25±0.03 |
| 1800 | 6.35±0.06 | 0.24±0.03 |
| 2000 | 4.12±0.06 | 0.19±0.02 |
| 2200 | 2.20±0.06 | 0.17±0.03 |
| 2500.00 | 1.01±0.05 | 0.14±0.03 |

### 3.4. Effect of pH and temperature on phenol degradation by *Paenibacillus mucilaginosus* AUN-AS01

The effects of factors such as pH values (5-11) and temperatures (20-45) on the degradation of phenol by *P. mucilaginosus* AUN-AS01 were investigated. The bacterial strain could grow within a range of pH 5–11 (Figure 2). Both acidic and

alkaline pH caused a marked inhibition of the phenol removal efficiency. Results also showed that the optimum pH for phenol degradation was 7.0 with the degradation rate 90.14±0.07. Similar phenomena were obtained by Karigar *et al*. (2006) for *Arthrobacter citreus* and Mohanty et al., (2017) for *Pseudomonas* sp.

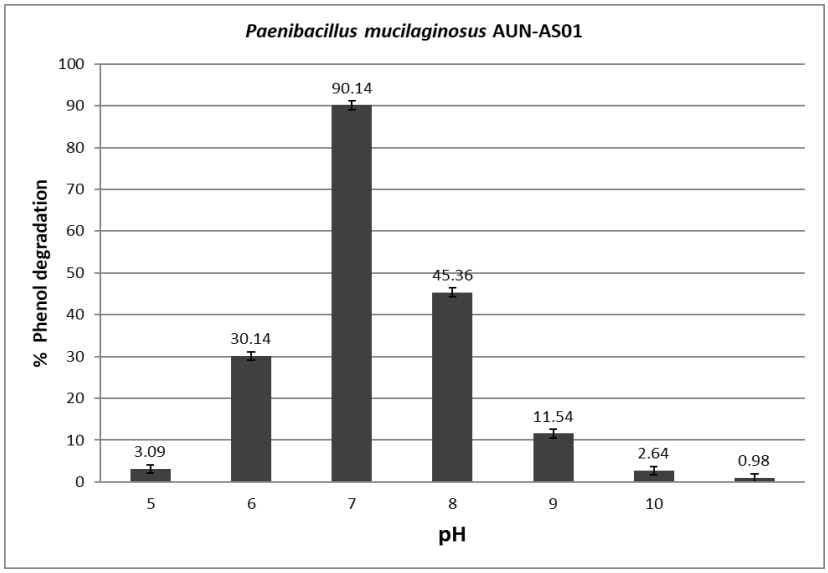


**Figure 2: Effect of pH on phenol biodegradation by the isolate *Paenibacillus mucilaginosus* AUN-AS01 at initial concentrations of phenol 800 mg/L$^{-1}$.**

### 3.5. Effect of Incubation temperature on phenol degradation by *Paenibacillus mucilaginosus* AUN-AS01

The results showed that the favored temperature for the bacterial growth of strain AUN-AS01 were ranged from 25–35 $^{o}$C (Figure 3) and the maximum degradation of phenol was 93.69±0.06 at the temperatures of 30 $^{o}$C (Table 3). Significant degradation also observed at 25 ºC (62.32±0.06) and 35 ºC (52.82±0.06) even if it was less than at 30 ºC. In addition to, degradation was inhibited both at low as well as high temperatures. Similar results have been reported on the *Pseudomonas pictorum* at 30 ºC by Annadurai *et al*. (2007), Cordova-Rosa *et al*. (2009); Mohanty et al., (2017) .Our results demonstrated that the strain could grow and remove more

than 90% of the phenol at the optimal conditions of 30 $^{o}$C , pH 7.0 and the initial concentrations of phenol 800 mg/L$^{-1}$ .

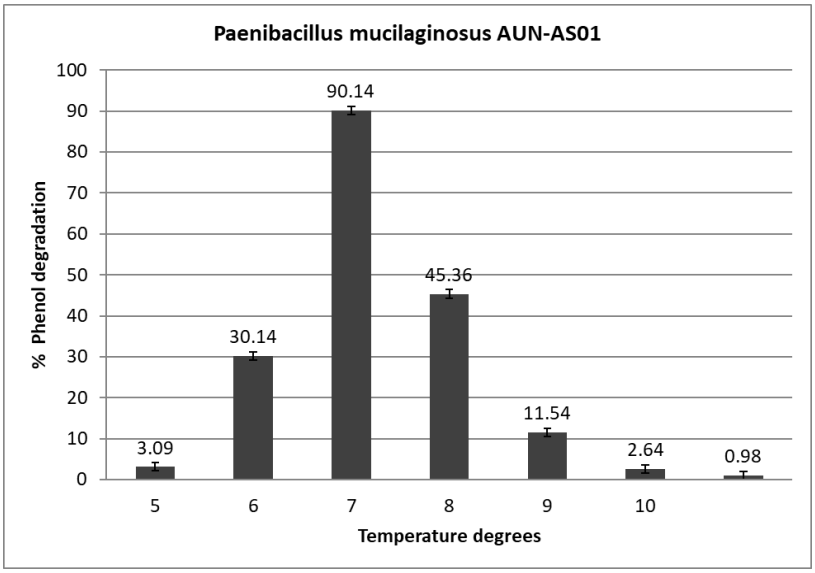


**Figure 3: Effect of temperature on phenol biodegradation by the isolate *Paenibacillus mucilaginosus* AUN-AS01 at initial concentrations of phenol 800 mg/L$^{-1}$.**

## Conclusion

In conclusion, a bacterial strain capable of degrading phenol was isolated from hospital wastewater in Assiut university, Egypt and it was identified based on 16S rRNA gene sequences and the phylogenetic analysis as *Paenibacillus mucilaginosus* strain AUN-AS01. The strain was reported to utilize phenol as the sole source of carbon and energy. Temperature, pH, and initial phenol concentration play key roles in determining the rate of phenol degradation by isolated bacteria. The optimal growth conditions for phenol degradation of the strain were at 30 °C , pH 7.0. and 800 mg/L$^{-1}$ initial phenol concentration that led to rate of phenol degradation above 90%. Our results demonstrate that, strain *P. mucilaginosus* AUN-AS01 could be recommended for phenol removal from hospital wastewater.

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# الملخص العربي

## العزل والتعرف الوراثي للبكتيريا المحللة للفينول الموجود بمياه الصرف الصحي بمستشفيات جامعة أسيوط

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يعتبر الفينول و مشتقاته من أكثر الملوثات شيوعًا في مياه الصرف الصحي بالمستشفيات وقد تم تأكيد آثارها المسببة للسرطان والسمية على الإنسان. إن تحديد الكائنات الحية الدقيقة الرئيسية التي تلعب دورًا في عمليات تحلل الملوثات أمر وثيق الصلة بوضع استراتيجيات المعالجة البيولوجية المثلى في البيئة. في الدراسة الحالية ، تم عزل سلالة بكتيرية سم يت AUN-AS01 من مياه الصرف الصحي في مستشفيات جامعة أسيوط عن طريق تقنية التخصيب في بيئة الأملاح المعدنية (MBS) والمضاف لها الفينول كمصدر وحيد للكربون والطاقة. تم تعريف هذه السلالة باستخدام تقنية PCR لتحديد تتابع جين 16S RNA الريبوسومي . وقد أكدت نتائج مقارنة هذا التتابع مع تتابعات الجين المتاحة في بنك الجينات GenBank وكذلك تحليل شجرة القرابة الوراثية لتعريف السلالة المعزولة باسم Paenibacillus mucilaginosus AUN-AS01 هذه السلالة كانت قادرة على النمو ولديها القدرة علي تحمل تركيزات من الفينول تصل إلى 1600 مجم / لتر. وقد لوحظ أن درجة الحرارة ، ودرجة الحموضة والتركيز الأولي للفينول تلعب أدوارًا رئيسية في تحديد معدل تحلل الفينول بواسطة السلالة AUN-AS01 . أظهرت النتائج أن السلالة كانت فعالة في إزالة 92.26 ± 0.05٪ من الفينول خلال 48 ساعة تحت الظروف المثلي والتي كانت عند تركيز اولي من الفينول 800 مجم / لتر ، وعند 30 درجة مئوية ورقم هيدروجيني 7.0. و تظهر نتائجنا أنه يمكن التوصية باستخدام سلالة P. mucilaginosus AUN-AS01 لإزالة الفينول من مياه الصرف الصحي في المستشف يات كمعالجة بيولوجية.