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Biodegradation of phenol by Pseudomonas putida strain B1isolated from Egyptian soil

Basma El-Sayed [1]*, Abdel-Fattah, G.M1 and Ali, M.S[2].

[1]*Botany Department, Faculty of Science, Mansoura University, 35516 Mansoura City, Egypt*

[2]*Microbiology Department, Faculty of Science, Ain Shams University, Egypt*

***Correspondent author**: baskal_2100@yahoo.com*

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Abstract: Bioremediation of toxic phenol is a cost-effective, efficient and eco-friendly method. The target of this research was to isolate potent degrading phenol bacteria, optimize the biodegradation and detect the pathway. A phenol degrading bacterium (B1) was isolated from acontaminated soil sample from amechanical shop, Cairo, Egypt. The isolate showed complete degradation of phenol as a sole carbon and energy source. The isolate was identified using 16S rRNA gene sequence and submitted to Genbank and identified as Pseudomonas putida strain B1 under accession number MT675100. The isolated strain exhibited the maximal and complete degradation of the phenol within 100 mg/l phenol concentration, initial pH of 7 and 30°C incubation temperature after 72 h incubation. P. putida strain B1 degraded phenol up to 800 mg/l. UV/VIS spectrophotometric analysis indicated that P. putida strain B1 degraded phenol via meta-ring cleavage pathway that revealed using the formation of 2-hydro-xymuconate.

keywords: Biodegradation, Phenol, Meta pathway, Pseudomonas

1.Introduction

Phenol and its derivatives are widely used in many industries such as pharmaceuticals, paint, textile, petrochemical, leather [1, 2]. Phenol pollution due to unprocessed effluents discharged by these industries are reported to pollute soil, groundwater and agricultural lands; and to harm the soil and plant health and productivity [3] as well as to affect on terrestrial and aquatic animals, and humans at very low concentrations. Phenol is listed as a priority pollutant by the U.S. Environmental Protection Agency and Agency for Toxic Substances and Disease Registry [4]. Phenolic compounds are highly soluble in water [5] with recalcitrant structure making it highly resistant to natural biodegradation [6, 7]. Several physio-chemical methods are widely assumed for the removal of phenol from industrial waste-waters [1, 2] but these methods are less efficient with high operation cost as well as produce secondary pollutants as intermediate compounds [8]. Therefore eco-friendly biodegradation showed a great reduction at low cost without the production of secondary pollutants to remove phenol and its derivatives from different industrial wastewaters [7, 9, and 10]. Several bacteria were recorded as phenol degraders either using anaerobic or aerobic metabolic activity as well as utilize it as a sole energy source [9, 12] such as *Pseudomonas* [7, 11, 13, 14, 15, 16, and 17]. Bacteria that degrade phenol follow either an ortho cleavage pathway that converts the catechol into an intermediate cis, sys muconic acid, or meta cleavage pathway that reduces the catechol to 2-hydroxyumunic cemaldehyde (2-HMSA) [15, 18].

This study aims to select and identify promising phenol degrading bacteria from polluted soil in Egypt, optimization of the degradation process, and detecting the degradation pathway was studied.

2. Materials and methods

1- Modeling and isolation of bacteria that degrade phenol

three Polluted soil samples the first one was collected from mechanical shop in Misr Elgdida, the second from mechanical shop in El-Zaytoon area the third from mechanical shop in El-Sawah square (Cairo,Egypt)

was collected in sterile containers in July 2019. Soil samples are cooled and stored in an ice box after collection and then transferred to a laboratory to separate them from germs. Soil sample was enriched by cultivation in Basal mineral medium (BMM) containing (g/L): $MgSO_4.6H_2O$, 0.912; NaH_2PO_4, 3.45; K_2HPO_4, 4.36; $(NH4)_2SO_4$, 1.26; Trace salts solution 1ml/l. The trace salts solution contained (g/100ml): $CaCl_2.2H_2O$, 4.77; $FeSO_4.7H_2O$, 0.37; $CoCl_2.6H_2O$, 0.37; $MnCl_2$, 0.10; $Na_2MoO_4.2H_2O$, 0.02) [19] supplemented with 100 mg/l phenol as a sole carbon source before incubation at 30°C for 72 h. Enrichment was done three times, then the last rich culture was 10 times more concentrated with sterile saline solution, and 100 µl from each dilution was produced under BMM agar (2%) medium supplemented with 100 mg / l phenol. Thereafter the installation of the plates was done at 30 ° C for 48 hours. After incubation, separate colonies were selected and cleaned.

2- Phenol degradation assay

The degradation and concentration of phenol were detect using the 4-aminoantipyrine colorimetric method according to the detailed procedure that was mentioned in Standard Methods for the Examination of Water and Wastewater [20]

3-Identification of selected bacterial isolate

The whole of the selected isolated bacteria was collected by the DNA extraction kit (Thermo; Fisher Scientific; USA) based on the manufacturer's instructions. The collected DNA was kept at 20^0C until the PCR reaction occurred. A pair of bacterial primer 27F (5′-AGA GTT TGA TCC TGG CTC AG-3R) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3 27) aimed to expand the 16S-rRNA-primer sites. used to build. Gene. 2 µl of bacteria transfer a portion of DNA through a 50 µl purified volume and form a PCR reaction. Maxima Hot Start PCR Master Mix (Thermo K1051) demonstrates PCR based on manufacturer's instructions. PCR was demonstrated on a genius model FGENO2TD thermal cyclist (Techney, England), and optimized for initial deformation at 95 ° C for 5 min, then at 95 ° C for 1 min 35 cycles, 54 ° C for 1 min, and 1 min for 72 At C, and eventually for 10 minutes at 72 C of genetic expansion. The enlarged genes were visualized with size markers on 1% agarose gel to indicate the size and purity of the products and then the enlarged bands were cleaned with a Geniget ™ PCR Purification Kit (Thermo K0701). The forward and reverse directions of the 16S rRNA gene were sequenced by the Gats Company (Constance, Germany) ABS 3730xl DNA sequencer. The footage obtained was reported by the BLAST Discovery Program, National Center for Biotechnology Information (NCBI), National Library of Medicine, USA [21]. These scenes were aligned by MEGA V6 software [22] and then exploded in Genbank, simultaneously downloading all closely related species and forming phylogenetic trees through neighborhood reach methods.

4. Optimization of phenol degradation

Factors influencing the degradation of phenol by the selected antibodies are performed by a single object and the parameters differ one by one. The test was performed in 250 ml bottles containing 100 ml BMM supplemented with 100 mg / l phenol infused with 1 ml of 50 x 105 CFU for selected bacterial separation before implantation at 30 ° C for 72 h. Biodegradation was carried out in triplicate using different; concentrations of phenol (100, 200, 300, 400, 500, 600, 700, 800 and 900 mg/l); different pH (from 5 to 11), different temperatures (25, 30, 37 and 40°C); different incubation period (24, 48, 72, 96 h). The appropriate parameters of one parameter were used to maximize the remaining parameters respectively**.**

5- UV–Visible Spectra of Intermediate Compounds

For the detection of intermediates,the sample was centrifuged to remove cells; the supernatant was scanned using 201 Scan UV–Visible spectrophotometer (Thermo Scientific). Spectra were recorded between 200 and 600 nm.

6- Statistical analysis

Statistical analysis for this study was done by Microsoft Excel 2010 software, Analysis

Toolpaek (Microsoft Corporation).The standard error (SE) for each data was sorted intoagraph.

3. Results and Discussion

Isolation and identification of Pseudomonas putida

Four colonies with different shapes were purified after soil enrichment. The bacterial isolate (B1) which exhibited complete degradation of phenol (100 mg/l) after 72h using the 4-aminoantipyrine colorimetric method was selected. Isolate B1 was identified by amplification and the sequenc of 16S rRNA gene as *Pseudomonas putida* (99% similarity respectively).

The nucleotide sequence was introduced to GenBank as Pseudomonas puta strain B1 under the MT7500 acquisition number. Neuber-indicated linking of phylogenetic tree with the alignment of 16S rRNA gene. *P. putida* strain B1was put in the phylogenetic cluster with many *Pseudomonas* sp. That approved the NCBI-BLAST results as well as the belonging of our strain to the genus *Pseudomonas* (Fig.1).

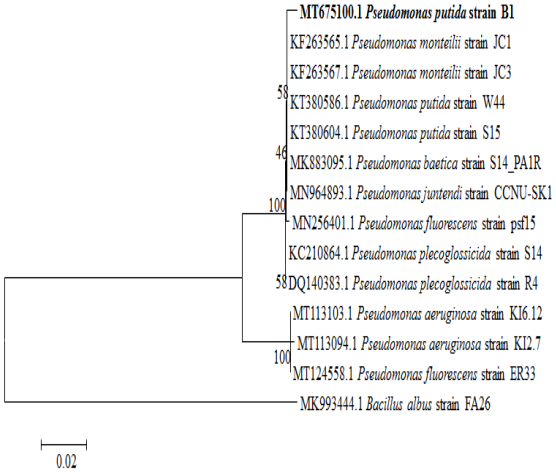

Figure (1): Neighbor-connecting phylogenetic tree of 16S rRNA genes.

2. Optimization biodegradation of phenol by P. putida strain B1

P. putida strain B1degraded phenol up to 800 mg/l with maximal and complete degradation of 100 mg/l phenol within pH 7 after incubation for 72 h at 30°C.

Increasing phenol concentration from 100 to 800 mg/l led to agradual decrease in the degradation percent from 100 to 13%. Further increase in phenol concentration inhibited phenol degradation by *P. putida* strain B1 (Fig. 2A).

The ideal pH for phenol degradation was observed in culture media of pH 7. Any modification in pH led to adecrease in the degradation percent of phenol using *P. putida* strain B1 (Fig. 2B).

The optimum temperature to degrade phenol was found to be at 30°C. Higher or lower incubation temperatures led to adecrease in phenol biodegradation percent (Fig. 2C).

Increasing the incubation period from 12 to 72 h led to agradual increase in phenol degradation percent by *P. putida* strain B1. No difference after further incubation for 96 h (Fig. 2D).

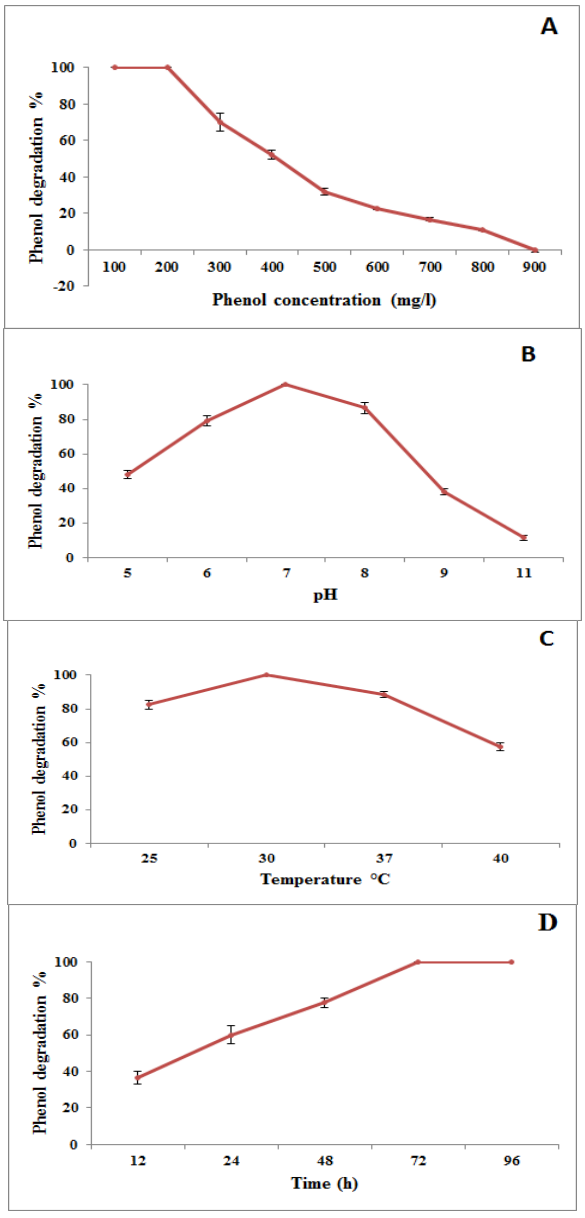

Figure (2): Effect of different phenol concentrations (A), pH (B), incubation

temperatures (C) and incubation periods on the degradation of phenol by *P. putida* strain B1. Error bars represent SE (n = 3).

3. Determination of phenol biodegradation pathway

UV/VIS spectrophotometric analysis showed the appearance of absorbance peak at 375 nm after 24 h incubation confirms the breakdown of the extradiol ring of phenol resulting in the formation of 2-hydro-xymuconate semialdehyde (2-HMS) confirming that *P. putida* strain B1degraded phenol via meta-pathway (Fig. 3)**.**

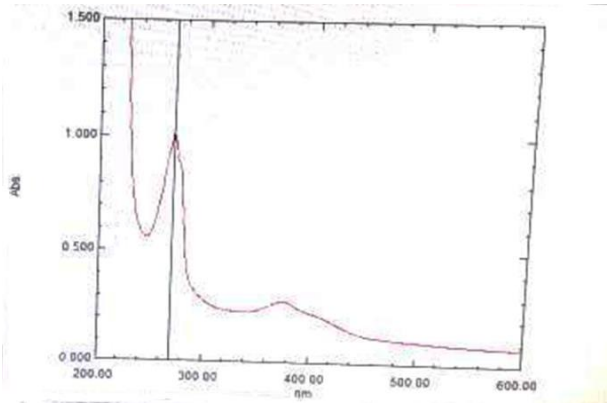

Figure (3): UV–Visible spectrum of Phenol biodegradation by *P. putida* strain B1 after 24 h incubation.

Discussion

Phenol pollution in water and soil resulted from industrial effluents is increasing which has the biggest toxic effects for all living organisms [3 and 15]. Bioremediation of toxic pollutants is a cost-effective as well as an environmentally safe way [10]

In this research, a polluted soil sample that was collected and enriched on BMM supplemented with phenol as a sole carbon and energy source for isolation of phenol degrading bacteria. Numerous work isolated phenol degrading bacteria from polluted sites [23, 24]. Isolated phenol degrading bacteria from Distillery effluent from Canna indica rhizosphere is grown in contaminated sites and the effluent of petrochemical wastewater, respectively.

The bacterial isolate (B1) degraded phenol (100 mg/l) completely after 72h was selected and identified using 16S rRNA gene sequencing as *P. putida* strain B1 (MT675100). *Pseudomonas* sp. Strain NBM11 and *P. putida* isolated as a phenol degrading bacteria by Mohanty and Jena (2017), Pazarlioğlu and Telefoncu (2005), respectively.

P. putida strain B1 was capable of degrading phenol up to 800 mg/l. This finding agreed to Vázquez-Rodríguez *et al.* (2006) who reported maximum phenol concentration tolerated by *P. putida was* 800 mg/l. Moreover, Bandhyopadhyay *et al.* (1999) and Mohanty and Jena (2017) recorded 1000 mg/l was the maximum phenol concentration tolerated by *P. putida.*

The optimum pH for phenol degradation by *P. putida* strain B1 was noted in culture medium adjusted to pH 7. This was comparable to the finding of Mohanty and Jena (2017) who found that maximal degradation of phenol by *Pseudomonas sp.* NBM11 within a range of pH 6.8 to 7.2 while pH 8 was the optimum for phenol degradation by *Acinetobacter Calcoaceticus* PA (Liu *et al.*, 2016).

In this research, the optimum incubation temperature for phenol degradation was 30°C. This was in accordance with the results of [4, 25, and 26] who reported that the optimum temperature of phenol degradation was 30°C by *Acinetobacter* sp. SA01, *Pseudomonas pictorum* and *Pseudomonas* sp. NBM11, respectively.

P. putida strain B1 degraded phenol (100mg/l) completely within 72 h and the increase in the initial concentration of phenol resulted in decrease in the percentage of degradation of phenol. Similar result was recorded by [4, 27], who reported that *P. putida* MTCC 1194 degrade phenol at 1000 mg/l concentration in 162 h.

P. putida strain B1degraded phenol via meta-pathway resulting in formation of 2-hydro-xymuconate semialdehyde (2-HMS) through the appearance of absorbance peak at 375 nm after 24 h obtained from UV–Vis spectra. This finding is in agreement with that of [28, 29] who illustrated that the increase and decrease in absorption at 268 and 375 nm in the spectra elucidate the cleavage of the ring of phenol resulting in the formation of 2-hydro-xymuconate semialdehyde (2-HMS) that indicated that *P. fluorescens* metabolized phenol in the meta-pathway through the aerobic process.

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