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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2021 18(2): 1294-1300. **OPEN ACCESS**

Biodegradation of phenol by *Pseudomonas aeruginosa* isolated from oil contaminated environments in Peru

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Hydrocarbons, phenolic compounds and their derivatives are toxic and carcinogenic. They cause adverse effects on human health and the environment. Through the years, the Peruvian jungle has been impacted by constant oil spills in populated areas, causing social conflicts involving the death of people. Unfortunately, there is a not technological solution available for their bioremediation. In this study, we isolated a new bacterial strain (QF50) from oil-contaminated soil collected in La Libertad (north of Peru). The 16S rDNA sequence alignment showed that this strain is *P. aeruginosa* DSM 50071. We show that QF50 at OD_{600} of 2.25 triggered substantial degradation of phenol (47.6 %) in 20 hours at pH 8 with 150 rpm of agitation. Our finding, demonstrates that phenol can be degraded by *P. aeruginosa* QF50 and provide a strong evidence that can be used as a biotechnological tool for rapid bioremediation of wastewater and soils contaminated with phenol in Peru.

Keywords: phenol, *Pseudomonas*, petroleum, biodegradation, biotechnology

INTRODUCTION

Many industries such as oil refineries, gas furnace industries, petrochemical plants, steel plants, phenolic resin manufacturing, plastics industries, textiles, and metallurgical operations, bring with them relatively common industrial wastes such as phenolic effluents (Duan et al. 2018). Currently, phenol has been listed in the top 20 hazardous and harmful chemicals by the International Maritime Organization (IMO) (Tornero & Hanke, 2016). In the Amazonian rivers of northern Peru, water contamination generated by oil extraction activities is very frequent and worrisome as they contain a complex mixture of dissolved inorganic and organic matter. Recently, high concentrations of phenolic compounds has been reported, which can potentially cause some kind of cancers and neurodegenerative diseases (Yusta-García et al. 2017).

Several methods are available for the removal of phenolic compounds from industrial effluents, the efficiency of these methods mainly depending on the time required for complete removal and the initial concentrations of phenol that will degrade (Nawawi et al. 2016). Various investigations have been carried out on physicochemical processes such as chemical oxidation, absorption, extraction, pervaporation, etc., to reduce phenolic compound pollutants in waste water (Pinto et al. 2005). However, these processes generally produce a

large amount of intermediate products and by-products, which occasionally tend to be more toxic than the original substrate and lead to secondary effluent problems to the environment or health, in addition, some processes can be more expensive and usually be a determining factor when choosing these types of treatments (Mishra & Kumar, 2017).

Biological phenol treatments are the most attractive and efficient methods, which overcome the main drawbacks mentioned in physicochemical methods; pure and / or mixed microbial strains are used in biological methods that degrade phenol and its derivatives into non-toxic products and is cost-effective (Wang et al. 2007). Currently, everything points in one way or another to the use of these biological methods since it implies greater sustainability to the environment during the treatment of phenolic compounds. There are different investigations on the variety of microorganisms that have been studied for their phenol degradation capacity. Also, Sepehr et al.(2019) reported cold-tolerant bacteria, isolated from alpine soils with phenol biodegradation capacity, identified as *Pseudomonas sp*, *Stenotrophomonas spp* and *Shinella spp*; at the same time, Patel et al. (2017) reported a yeast such as *Rhodosporidium kratochvilovae* HIMPA1 that degrades up to a concentration of 1000 mg-L of phenol.

In the present research, the objective was to determine the influence of pH and stirring speed on the biodegradation of phenol by *Pseudomonas spp*. isolated from oil contaminated environments in Peru.

MATERIALS AND METHODS

Sampling

Samples of oil-contaminated soil were collected in the region of La Libertad, Peru (7º48'28''S, 78º03'39''W) (Figure 1) using a simple sampling technique. Briefly, two kg of soil was collected from one site in an autoclaved flask, samples were immediately stored at 4 ºC and transported to the laboratory for bacterial isolation.

Bacterial isolation

Bacteria were isolated on *Pseudomonas* broth (PB) containing glutamate 1%, peptone 2%, magnesium chloride 0.14% and potassium sulfate 1%. We used 5 gr of soil in 100 ml of PM the mixture was incubated at 35 ºC for 48 hours and then were reseeded in a petri dish containing agar PM. Plates were incubated at 35 °C for 48 hours. Bacterial colonies with a characteristic pigmentation of *Pseudomonas* were then isolated to obtain pure cultures and stored at 4 ºC for later use (Ghaima et al. 2018).

Molecular characterization and phylogenetic analysis

Extraction of the genomic DNA was performed using thermal lysis and the 16S rRNA gene fragment was amplified by polymerase chain reaction (PCR) according to the methodology of Sernaque-Aguilar et al. (2019) using the universal 16S-27F rRNA oligonucleotides (5'- AGA GTT TGA TCC TGG CTC AG -3 ') and 16S-1492R rRNA (5'-AAG GAG GTG ATC CAG CCG CA -3'). Then, molecular analyzes of the 16S rRNA gene were performed using next generation sequencing (Ion Torrent, MR DNA). For phylogenetic analysis, the 16S rDNA sequences were aligned using BioEdit and the phylogenetic trees were constructed with Mega X software (Kumar et al. 2016). The sequences of the isolated *Pseudomonas*-type strains used for the analysis were retrieved from the Gen Bank database of the National Center for Biotechnology Information (NCBI) and EzBioCloud (Yoon et al., 2017).

Conditioning the phenol solution preparation

A chemically pure phenol solution (Merck, USA) was prepared at a concentration of 10 gr/L in NaOH 0.02M (Li et al. 2010a).

Preparation and adaptation of the bacterial inoculum

P. aeruginosa grew up in 100 ml of BHI broth pH 7 (BD Difco™), at 35 °C for 24 hours. Bacteria cells were collected by centrifugation at 7000 rpm for 15 min and suspended in sterile saline solution (0.85% NaCl). The mixture was adjusted to an optical density of 0.7 in sterile saline at 600 nm by using the Uv-vis Spectrophotometer, Evolution™ 260 Bio. Adapation of of *P. aeruginosa* to phenol was carried out in BHI broth containing 50 mg/L of phenol. The prepared inoculum (2% v/v) was incubated at 35 ºC for 48 hours. Once bacterial growth was obtained in 50 mg-L of phenol from, it was transferred in a broth containing 100 mg-L of phenol and so on until reaching 600 mg-L of phenol (Ajaz et al. 2004).

Figure 1: Location of the sampling site in the region of La Libertad, Peru.

Phenol biodegradation assay

A multi-level factorial design was performed with two factors, pH (6, 7 and 8) and stirring speed (100, 150 and 200 rpm). The tests were carried out in triplicate of each base run, adding a total of 27 experiments. 2000 ml of BHI broth was prepared and 50 ml were dispensed into previously sterilized precipitation flasks. Subsequently, it was completed with the phenol solution until reaching an initial concentration of 600 mg-L. Then, 2 % (v/v) of the already prepared *P. aeruginosa* inoculum from a young culture of 48 hours of growth, previously adapted, was added. It was incubated at 30 ºC for 20 h and every 4 hours the residual phenol concentration and bacterial growth were monitored (Tebbouche et al. 2016).

Analytical methods

Bacteria cells concentration was determined by analyzing the optical density at 600 nm using a UV/Vis spectrophotometer (Evolution™ 260 Bio) (Li et al., 2010b). Determination of the phenol concentration was performed by high performance liquid chromatography using UHPLC Thermo Scientific (Dionex Ultimate 3000) with DAD UV/Vis detector (Patel et al. 2017).

Statistical analysis

All the experiments were carried out in triplicate, the data obtained from the residual phenol concentration and the cells concentration were analyzed by analysis of variance (ANOVA) using the Minitab 18 computer software package (Song et al. 2009)

RESULTS AND DISCUSSION

This study focused on the isolation and evaluation of the capacity of *P. aeruginosa*, isolated from a soil contaminated with crude oil, for the biodegradation of phenol. Six microbial cultures were obtained, all were analyzed to determine the degradation of phenol in liquid medium and the isolated *P. aeruginosa* QF50 was the most active in the degradation of phenol. Bacterial identification was carried out by PCR amplification, which obtained a fragment of the 16s rRNA gene of 1334 bp from the QF50 strain, in the same way the non-amplification of the extraction control (CE) and the PCR (C-), indicates that both processes were carried out without contamination (Figure 2). Searching by BLASTN of this sequence indicated that the QF50 strain presented a 99.62 % similarity with *P. aeruginosa* DSM 50071. Therefore, based on the phylogenetic tree constructed with the similarity of 16S rRNA (%) (Figure 3), the QF50 strain was identified as a *P. aeuginosa*.

Phenol degradation and cell growth were determined (OD_{600}) of *P. aeruginosa* QF50 at various conditions of pH and stirring speed of the fermentation medium (Figure 4-5). Microbial growth under all conditions was stable in OD_{600}= 0.5 - 2.5.

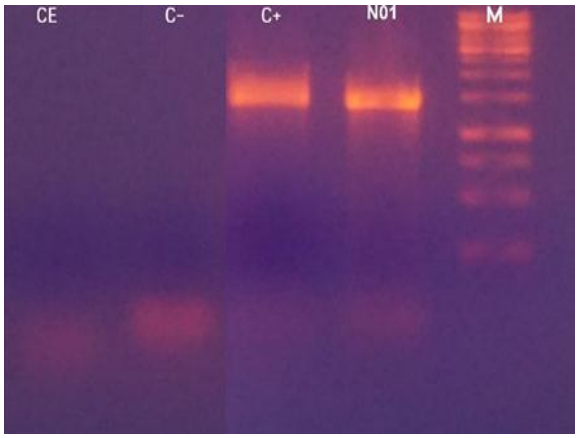

Figure 2: Agarose gel electrophoresis of the 16S rRNA gene PCR amplified from the isolate (sample N01). CE: Extraction Control, C-: Negative Control, C +: Positive Control (*Escherichia coli* ATCC 25922), M: 1Kb marker.

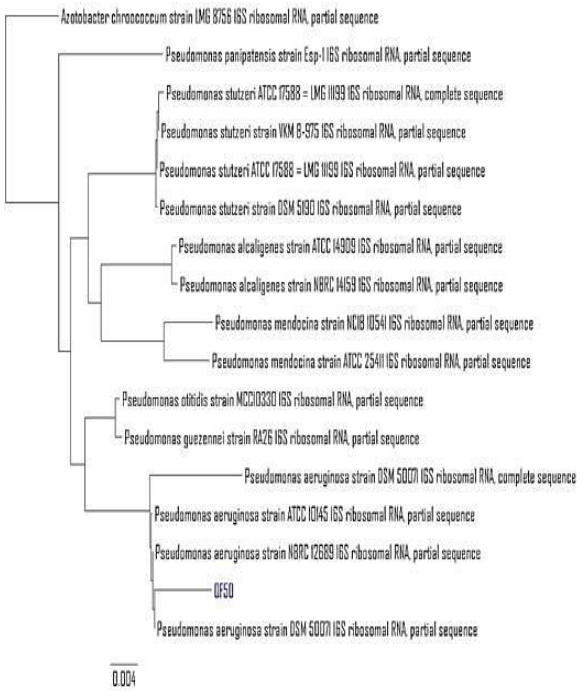

Figure 3: Phylogenetic analysis of 16S rRNA sequences of the bacterial isolate QF50. The tree was constructed using sequences from comparable regions of the 16S rRNA gene sequences that are available in public databases. The analysis was carried out with MegaX.

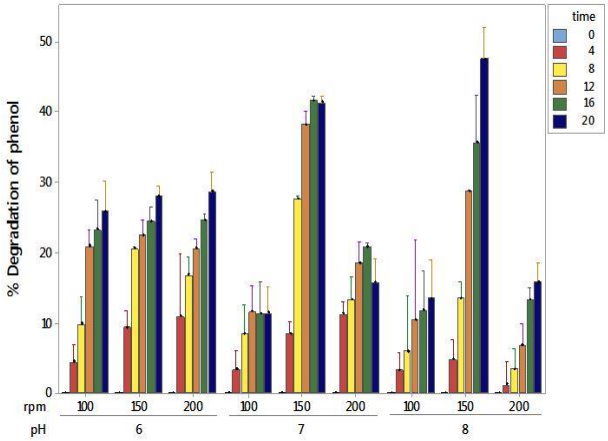

Figure 4: Effect of pH and stirring speed (rpm) on phenol degradation *of P. aeruginosa* QF50 for 20 h at room temperature.

The Figure 4 shows that *P. aeruginosa* QF50 when it grows to OD_{600}= 2.25 during 20 hours incubating at 30 ºC, pH 8 at 150 rpm, achieves biodegrade 47.60 % phenol, but to pH=7, 150 rpm and OD_{600} = 2.24 is obtained 41.41 % of biodegradation, however by varying the stirring at 100 rpm, OD_{600}= 2.21 and the pH=7 drastically the biodegradation to 11.47 % (Figure 4). This results differences significant between pH factors and stirring speed with respect to biodegradation of phenol, with a *p* value = 0.018 and a significance level ($p<0.05$). In turn, the interaction of time with pH and stirring speed presented p= 0.303 and p= 0.008 respectively.

P. aeruginosa QF50 in the presence of phenol (600 mg-L) proliferates at high biomass densities (OD_{600}~ 2-2.5), where at the end of the process it was observed that the cultures were very viscous and dense (Images not shown). According to Benedek et al. (2020), *Pseudomonas spp.* has a high capacity to generate biofilms in liquid cultures, which allows the development of high viscosities, this would explain the adaptation mechanism of the QF50 strain to be able to metabolize compounds such as phenol so as not to affect the cell population development, producing biofilms, optimized by the conditions of alkalinity (pH 8.0) and stirring (150 rpm) having a phenol biodegradation yield close to 50 % in almost 20 hours of bioprocess (figure 4).

Figure 5 describes that *P. aeruginosa* QF50 after 20 hours of bioprocess at pH 8 presents similar amounts of biomass (OD_{600} ~ 2.1), in all conditions of agitation (100, 150 and 200 rpm), but exhibits a drastic change in its phenol biodegradation activity (Fig. 4) dependent on agitation, where at 100 and 200 rpm it only

biodegrades 13.46 % and 15.83 % respectively, while at 150 rpm it is 47.6 %, here it is observed that the QF50 strain has 3.24 times the biodegradation capacity of phenol, the factor for this result is the analysis of the specific growth rate reached at 150 rpm μ_{150}=53 x 10^{-3} h^{-1}, was double that at 100 and 200 rpm with growth rates μ_{100}=20 x 10^{-3} h^{-1} and μ_{200}=24 x 10^{-3} h^{-1}, respectively (data not shown).

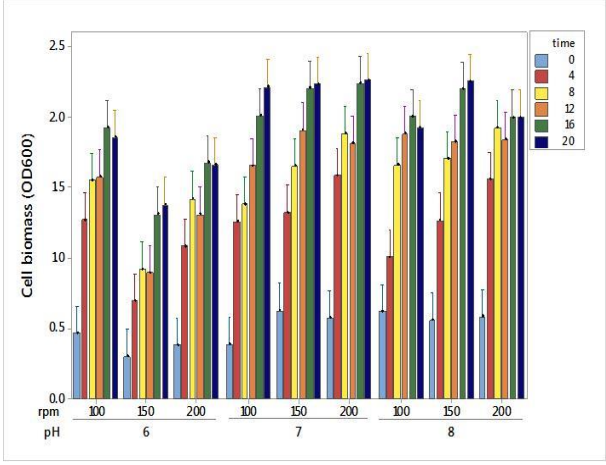

Figure 5: Effect of pH and stirring speed (rpm) on growth (OD_{600} cell biomass) of *P. aeruginosa* QF50 for 20 h at room temperature.

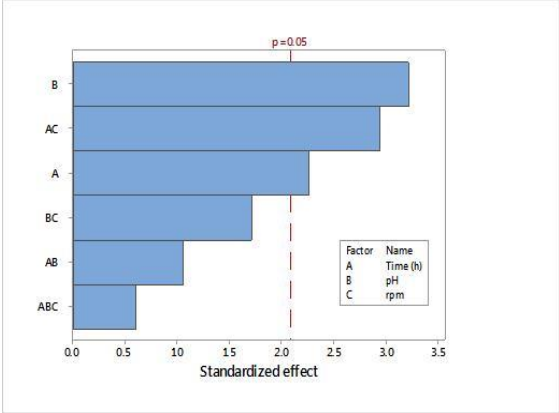

Figure 6: Pareto diagram of main standardized effects and interaction of the factors (time, pH and stirring speed) with respect to the percentage of biodegradation of phenol. The vertical line represents the limit between significant and non-significant effects with a 5% risk of error.

On the Pareto diagram (Figure 6), the significant effects of time, pH and stirring speed are evaluated, which were found to have 95% of the confidence level in the experimental domain studied ($p<0.05$); in turn, pH was the main variable with a statistically standardized effect of 3.2 relative to phenol degradation (Holland et al., 2019), within our experimental model with an R2 of 95.25%.

Here we show (Figure 4-5) that pH was the most important variable that allowed greater degradation of phenol by *P. aeruginosa*, which is consistent with previous investigations. Lin et al. (2010) showed that the influence of pH on the degradation of phenol may be due to its effects on transport stimulating enzymatic activities and nutrient solubility. Many previous studies indicated that the optimum pH for growth and phenol degradation of *Pseudomonas spp*. was in the 7-10 range and depended on bacterial origin (Moghadam et al., 2016). However, research has shown that each strain shows greater phenol biodegradation at pH. For example, the best pH ranges for the biodegradation of phenol by *P. aeruginosa* KBM13 was at pH 8, presenting a removal of 92% in an initial concentration of 500 mg-L of phenol for 48 h, under stirring conditions of 120 rpm (Ghaima et al. 2018); while, *Ewingella americana* was able to degrade optimally at a pH of 7.5 and a stirring speed of 200 rpm (Khleifat, 2006). Therefore, pH significantly affects the biochemical reactions that occur in the degradation of phenol, research indicates that pH influences the surface charge of cells (Khleifat, 2006). On the other hand, the agitation speed is not proportional to the percentage of biodegradation, due to this it is important to know that *P. aeruginosa* is a facultative anaerobic organism that does not require a high oxygen transfer to grow; likewise, Asshifa et al. (2017) reported that a *P. aeruginosa* strain preferred the microaerobic condition under low conditions of stirring speed, for growth and the formation of metabolites in the presence of hydrocarbons. These findings will allow us to maximize the biodegradation rate in future work considering other variables that affect the process. Consequently, the results of Figures 4 and 5 make it known that the *P. aeruginosa* QF50, isolated from oil-contaminated soil in Peru, is one of the strains that faster (20 hours) and plus efficiently (~50 % biodegradation) they can degrade phenol.

CONCLUSION

In this research it is described that *P. aeruginosa* QF50 isolated from soil contaminated with oil, has the ability to grow in phenol (600 mg-L) at different pH conditions and stirring speed, managing to degrade the to the 47.60 % when incubated by 20 hours, 150 rpm and at pH 8. Therefore, *Pseudomonas aeruginosa* can be used as a model system for the biodegradation of phenolic compounds and optimize its performance

in future research, as well as being applied in the bioremediation of wastewater effluents and soils contaminated with phenol.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

The authors acknowledge the infrastructure and support of the Laboratorio de Investigación de Tecnologías Limpias y/o Emergentes of the Facultad de Ingeniería of the Universidad Nacional de Trujillo.

AUTHOR CONTRIBUTIONS

JCM designed the experiments and performed the chemical analyzes. CQ performed the laboratory experiments, data analysis and editing of the manuscript. JBS conducted the field experiments. DUV and ME performed data analysis, editing and revision of the manuscript. All authors read and approved the final version.

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