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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, 2017 14(4): 713-720. **OPEN ACCESS**

Biodegradation of phenol by *Pseudomonas aeruginosa* isolated from soil contaminated with diesel fuel

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Phenolic compounds are toxic at low concentrations for flora and fauna. Because of its toxicity, there is a need to decontaminate the soils polluted with these compounds. The aim of this study was to investigate the effect of pH, temperature and nitrogen source on phenol degradation by *Pseudomonas aeruginosa*. Nineteen isolates of *P. aeruginosa* were isolated from contaminated soil with diesel fuel on minimal medium containing phenol (1g/L) as carbon source. All the isolated bacteria were tested for phenol degradation in liquid medium and the isolate *P. aeruginosa* KBM13 was the most active in phenol degradation. This isolate was selected and tested for the ability to use phenol as carbon and energy source. The strain was efficient in removing 92 % of the initial concentration 500 mg/L phenol within 48 h, and had a tolerance of phenol concentration as high as 1400 mg/L. Phenol was degraded rapidly at pH (7 to 9), but the maximum rate of phenol degradation by *P. aeruginosa* was at pH 8. Also, the maximum phenol degradation was observed at 40˚C. Among all nitrogen sources, yeast extract was found to be the best for growth and phenol degradation in our study. In conclusion, these results indicated that *P. aeruginosa* KBM13 possesses a promising potential in treating contaminated soil and water from phenol pollution

Keywords: *Pseudomonas aeruginosa*, Phenol, Biodegradation

INTRODUCTION

Phenol and phenolic compounds are important for many industries, therefore, a large amount of phenol are effused into stream and soil, and are widely distributed as pollutants. Phenol is toxic to most organisms and potentially carcinogenic to humans (Wallace, 1996). The phenolic compounds are contaminants in environmental matrices, food, and medicine products that can easily be absorbed through humans and animals' skin or mucous membranes. Phenol inhibits microbial activity due to membrane damage at higher phenol concentrations. Degradation of phenol is of great importance due to their toxicity and high solubility (Ruiz-Ordaz et al. 1998). Physicochemical methods, such as ozonation, ultra violet, hydrogen peroxide, Fenton's reagent have been employed to eliminate phenol in the polluted samples (Lin and Chuang, 1994). These methods were effective in removing phenol but were complex and costly. Alternative methods for the biodegradation of phenols are environmentally friendly and cost effective technologies preferred to reduce phenol compounds (Tay et al. 2005). Numerous bacterial species have been isolated and characterized as phenol-degrading microorganisms through the utilizing phenol as the sole source of carbon and energy (Geng et al. 2006). Many microorganisms have pathways for the aerobic degradation of phenol by oxygenation of phenol by phenol hydroxylase enzymes to form catechol, followed

by ring cleavage adjacent to, or in between, the two hydroxyl groups of catechol (Afzal et al. 2007). The optimization of growth conditions for phenol degradation is requiring for the development of the bioprocess. In general, optimization studies involving minimize the error in determining the effect of parameters and the results are achieved in an economical manner (Abdel-Fattah et al. 2007). The aims of this study were to isolate and identify *Pseudomonas aeruginosa* from diesel polluted soil with potential activity for phenol degradation and investigate the effect of different conditions on the degradation such as pH and temperature

MATERIALS AND METHODS

Isolation of bacteria

Thirty soil samples from diesel polluted regions in Baghdad, Iraq, were collected. One gram from each soil sample was placed in 9 ml of asparagine broth enrichment medium consisting of 2 gm/L asparagine L-monohydrate (BDH, England), 1 gm/L K2HPO4 (BDH, England) and 0.5 gm/L MgSO4.7H2O (BDH, UK) in order to enhance *Pseudomonas* growth. The samples were incubated for 48 h at 37ºC with shaking at 120 rpm (Al-Hinai *et al.* 2010). A loopful of the resulting bacterial suspension was streaked directly onto the King A and King B media and incubated at 37ºC until colonies developed. After blue colony apparition in the King A medium and the red colony In the King B medium, *Pseudomonas aeruginosa* bacterium were identified according to the key of Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). The identification was further confirmed by using a Vitek-2 system (bioMérieux).

Growth determination of phenol degrading isolates

To study the extent of degradation, the cells were grown in a Minimal Salts (MS) medium with the following composition: Phenol 1gm/L; K_2HPO_4, 1.5 gm/L; KH_2PO_4, 0.5 gm/L; (NH4)2SO4, 0.5 gm/L; NaCl, 0.5 gm/L; Na2SO4, 3.0 gm/L; Yeast extract, 2.0 gm/L; Ferrous sulfate, 0.002 gm/L; $CaCl_2$,0.002 gm/L in conical flasks containing and inoculated with *P. aeruginosa* isolates. The experimental studies were carried out in shake flasks with agitation at a rate of 120 rpm, temperature at 32^{o}C. Bacterial growth was determined in terms of cell mass by measuring optical density at a wavelength of 600nm.

Effect of Initial Concentration on Phenol Biodegradation

To study the effect of the initial concentration of phenol on the biodegradation by *Pseudomonas aeruginosa*, we prepared phenol solutions in MS medium to different concentrations of 100, 200, 400, 500, 600, 800, 1000, 1200, 1400 mg/L. Phenol was supplemented in the media as the sole carbon source. After that, volume of every solution was 50 ml, which we mixed with $1.5*10^8$ UFC/ml of *Pseudomonas aeruginosa* and submitted to agitation and incubation for intervals 24 h, 32 h and 48 h.

Effect of temperature of the medium on phenol degradation

P. aeruginosa cells were grown in MS medium with 500 mg/L of phenol at different temperature values (20^{o}C, 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C, 45^{o}C) at pH 7 and inoculum size 5 %v/v. This mixture was contained in 250 ml Erlenmeyer flasks. The cultures were placed on a shaker (120rpm) at the above temperatures. At different times, growth and phenol degradation were measured.

Effect of pH on phenol degradation

The effect of pH (5-10), on phenol degradation was tested. Cells were grown as shake cultures at 40^{o}C in MS medium supplemented with 500 mg/L phenol and inoculum size 5 %v/v in 250 mL Erlenmeyer flask. At different times, growth and phenol degradation were measured.

Effect of Nitrogen source on the growth and phenol degradation

Effect of different nitrogen source were determined in the previous medium in which nitrogen source was replaced with Peptone, Yeast extract, $NaNO_3$, Urea and Valine at concentration of 2 g/L. The used positive control was NH_4Cl. After 2 days at 40^{0}C, bacterial growth and phenol degradation were determined.

Estimation of phenol

The phenol concentration in the sample was determined by using 4-aminoantipyrine method (Folsom et al. 1990). 10 ml of reaction mixture containing 9.8 ml of distilled water, 100 µl of sample, 25 µl of 2% 4-aminoantipyrene and 50 µl of 2 M ammonia were mixed well and added 25 µl of 8% potassium hexacyanoferrate (III). The

absorbance at 500 nm was measured and compared with phenol standards

RESULTS AND DISCUSSION

This study was focused on isolation and evaluation of the capability of *P. aeruginosa*, isolated from diesel fuel polluted soil, for phenol degradation. Polluted soil samples were collected from different regions in Baghdad, Iraq for bacterial isolation on minimal salt agar medium, containing phenol (1g/L) as carbon source. The identification of bacteria was conducted by using selective media and Vitek-2 system (*bioMérieux*). Out of 30 soil samples; nineteen isolates of *P. aeruginosa* were obtained and screened for phenol degradation. All the isolated bacteria were tested for phenol degradation in liquid medium and the isolate *P. aeruginosa* KBM13 was the most active in phenol degradation.

The phenol degradation and cell growth at OD600 of *P. aeruginosa* KBM13 at various initial phenol concentrations were determined. The maximum biomass and degradation of phenol were observed at the initial phenol concentration of 500 mg/L (Figure 1). An inhibitory effect showed that the biomass growth and the degradation of phenol were declined with the elevated initial phenol concentration higher than 500 mg/L. The strain was efficient in removing 92 % of the initial concentration 500 mg/L phenol within 48 h. The removal rates of phenol were above 80 % at the initial phenol concentration ranging from 400 to 800 mg/L, while there was no growth of phenol-degrading bacteria when the initial phenol concentration was higher than 1400 mg/L. This observation was in good agreement with that of Kotresha and Vidyasagar (2014) who reported that the strain of *Pseudomonas aeuriginosa* MTCC 4997 isolated from effluents collected from petrochemical industries utilized phenol as a sole source of carbon and energy, capable of degrading phenol up to 1400 mg/L. The strain KBM13 could grow on phenol up to a concentration of 1000 mg/L with the degradation rate of 46.61%.

It was obvious that phenol biodegradation by the strain KBM13 is strongly dependent on the initial phenol concentration due to toxic effects induced by the substrate. The effect of initial phenol concentration when we use phenol as substrate in phenol biodegradation is particularly important because phenol, especially at higher concentrations, is an inhibitory material for microbial cells (Al-Khalid and El-Naas, 2012).

The effects of factors such pH values and temperature on the phenol degradation were investigated. The bacterial strain *P. aeruginosa* KBM13 could grow within a range of pH 5–11 (Figure 2) and the degradation of phenol was above 83.64 % in the range of pH 7–8. The optimum pH for phenol degradation was 8.0. Many previous studies indicated that the optimum pH for the growth and phenol degradation of *Pseudomonas* spp. was in the range 7-10 and it's dependent on the bacterial origin (Sarnaik and Kanekar, 1995, Shahriari et al. 2016). Studies have shown each strain shows the best phenol biodegradation at certain pH. For instance, the best pH ranges for the biodegradation of phenol by *Halomonas campisalis* was determined between 8 and 11, while the best condition for *Klebsiella oxytoca* was 6.8 (Khleifat, 2006).The effect of pH in phenol degradation which may be because of its effects on transportation, stimulating the enzymatic activities and the nutrient solubility (Lin et al. 2010). The pH significantly affects the biochemical reactions required for phenol degradation. Reports indicate that the pH affects the surface charge of the cells of the activated sludge biomass (Aksu and Gonen, 2004).

Temperature exerts an important regulatory influence on the rate of metabolism and enzymes. The results showed that the bacterial growth of strain KBM13 and the degradation of phenol (above 80%) were favored at temperatures of 35^{o}C - 40^{o}C (Figure 3). The biomass and phenol degradation reached the maximal values at a temperature of 40^{o}C, On the contrary, the phenol degradation declined sharply when the temperature reached 45^{o}C and over. Therefore, the optimal temperature for the growth of *P. aeruginosa* KBM13 was 40^{o}C.

Among the other environmental conditions, temperature plays an important role in affecting petroleum hydrocarbons biodegradation and when the temperature increase, the bacterial metabolism increases as well (Mohn and Stewart, 2000). In this work, temperature showed significant effect on phenol biodegradation at the temperature above 30 ºC and the optimum was 40 ºC (Figure 3). According to the source of isolation, this strain revealed variable in optimum temperature for phenol degradation in comparison with other studies reported previously who mentioned that the optimum temperature was 30 ºC (Reda and Ashraf, 2010, Shahriari et al. 2016).

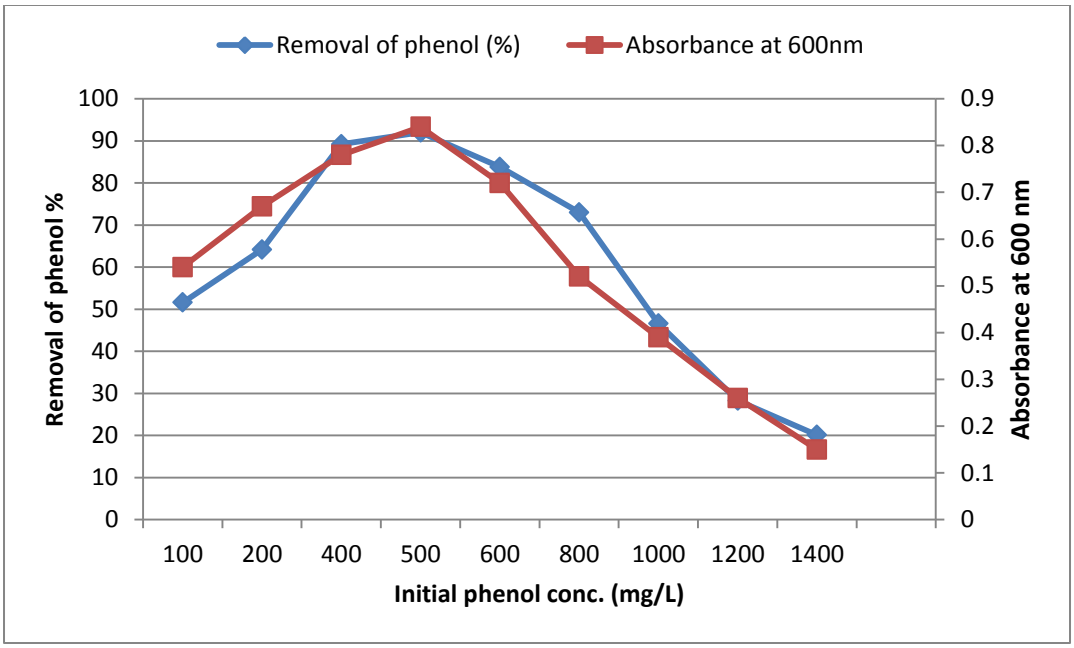

Figure 1. Profile of *P. aeruginosa* (KBM 13) growth and phenol degradation at various initial concentrations of phenol.

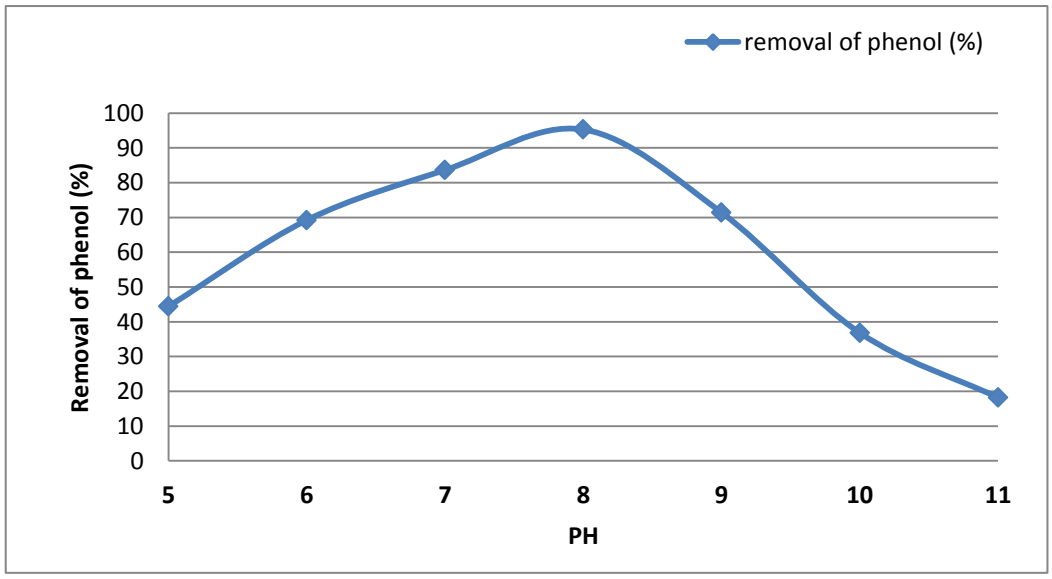

Figure 2. Effect of different pH values on the biodegradation of phenol by *P.aeruginosa* (KBM 13).

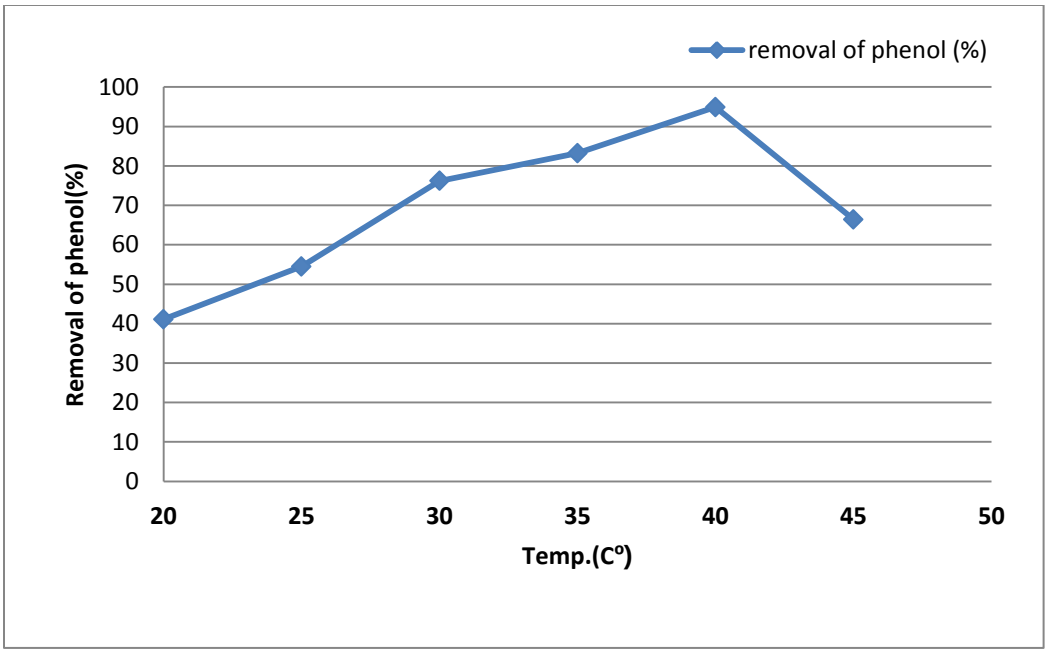

Figure 3. Effect of temperature on the biodegradation of phenol by *P.aeruginosa* (KBM 13).

Also, one of the studies demonstrated that the strain *Pseudomonas aeuriginosa* MTCC 4997 isolated from effluents collected from petrochemical industries exhibited a complete degradation of phenol at wide temperature from 15°C to 45°C with an optimum of 37°C (Kotresha and Vidyasagar, 2014). Bioavailability and solubility of less soluble hydrophobic substances, such as aliphatic and polyaromatic hydrocarbons, are temperature dependent. A temperature influenced the viscosity, thereby affecting the degree of distribution and an increase or decrease in diffusion rates of organic compounds (Rajani and Reshma, 2016).

The effect of six organic nitrogen sources (Peptone, Yeast extract, $NaNO_3$, Urea, NH_4Cl and Valine) at concentration of 2 g/L on the growth and phenol degradation by *P. aeruginosa* was studied. The nitrogen sources Were supplemented to MS medium containing 500 mg/L phenol and incubated at 40°C in a temperature controlled orbital shaker at 120 rpm and pH 8 for 48 hrs in triplicates and the results were shown in Figures 4 and 5. It was observed that the growth of *P. aeruginosa* and phenol degradation affected by the type of nitrogen source clearly. Hence, among the nitrogen sources tested, yeast extract was the best source for maximum growth and phenol degradation. The percentage phenol degradation with the addition of yeast extract was 91.36%. While the nitrogen source, Valine gave the lowest growth and phenol degradation (26.8%). The present study indicated that NH_4Cl influences the rate of phenol degradation but at a lower rate on the growth in contrast with the activity of peptone. In general, the nitrogen sources, yeast extract, NH_4Cl and peptone exhibited high rates for growth and phenol degradation (more than 70%).

It is well-known that nutrients, particularly nitrogen and phosphorus, are required for to improve the aromatic compounds biodegradation. The current study demonstrated that the addition of yeast extract in the medium showed highest phenol removal efficiency of 91.36 % and it also enhanced the growth of the strain *P. aeruginosa* KBM13. This might be due to the structure of yeast extract as it is readily available as amino acids in the mineral salts medium which essential to phenol degradation process by bacterial cells. The addition of nutrients (N/P) might be effective in increasing the biodegradation of organic compounds because these amendments effectively stimulate bacterial growth (Ochieng et al. 2003, Walecka-Hutchison and Walworth, 2006).

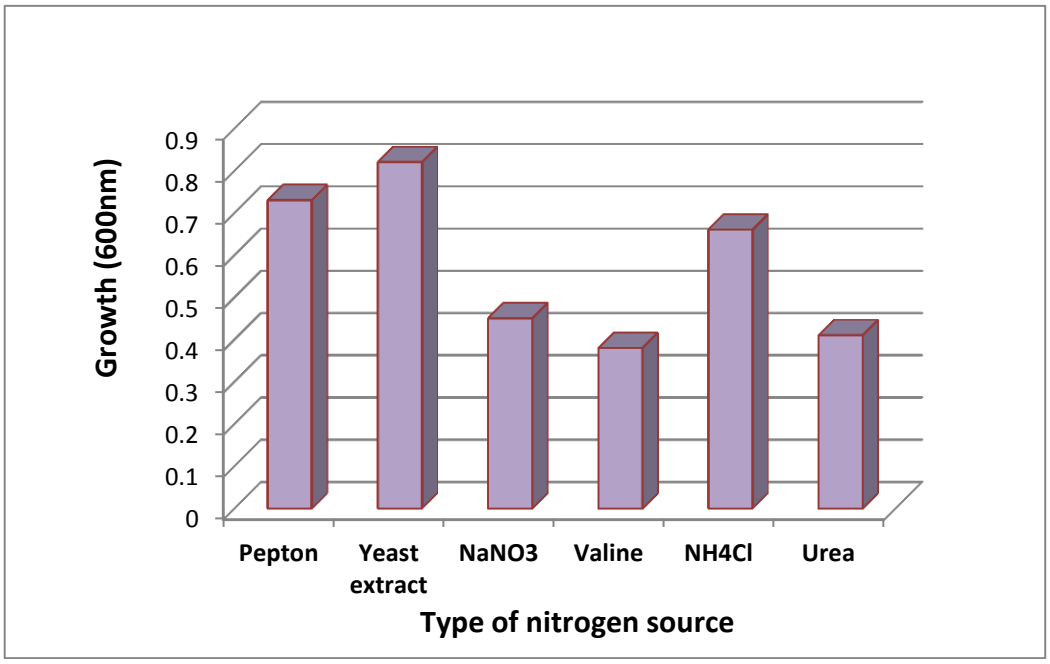

FIGURE 4. Effect of different nitrogen sources on the growth of *P.aeruginosa* (KBM 13).

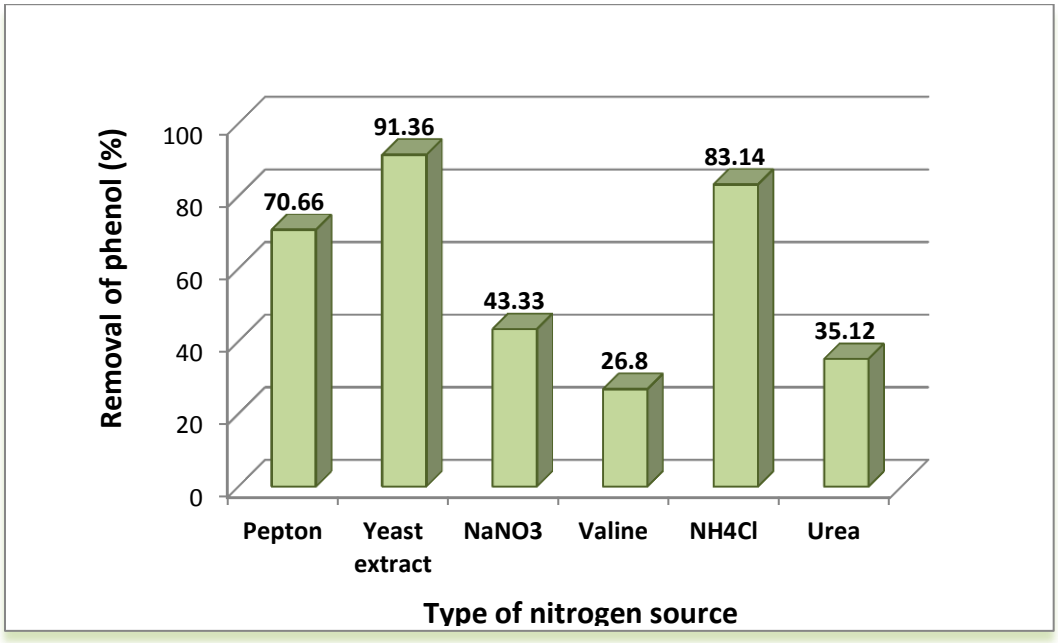

Figure 5. Effect of different nitrogen sources on phenol degradation by *P.aeruginosa* (KBM 13).

CONCLUSION

In conclusion, a bacterial strain *P. aeruginosa* KBM13 isolated from soil contaminated with diesel fuel has the ability to grow in a liquid medium with phenol at different concentrations as the sole carbon and energy source. The strain was able to degrade 92 % of the initial concentration 500 mg/L phenol within 48 h and grow at the phenol concentration of as high as 1400 mg/L. The optimal growth conditions for phenol degradation of the strain were at 40°C, pH 8.0 and yeast extract as optimal nitrogen source. The results revealed the significant ability of the strain KBM13 to consume phenol. Obviously, this strain is able to be seen as an important tool on bioremediation of wastewater effluent, and soil contaminated with phenol.

CONFLICT OF INTEREST

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

ACKNOWLEGEMENT

This work was supported by Biotechnology department, Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of the experiments. KKG performed the experimental work, BSR and MMM carried out laboratory tests. KKG wrote the manuscript, all authors revised and approval the final version.

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