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Effect of physicochemical factors on the biodegradation of phenol by *Pseudomonas putida* ATCC 12842 and *Pseudomonas fluorescens* ATCC 948

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Phenol is a very toxic substance and it can cause a number of environmental problems when it enters a water system. Biological treatment is considered a cost-effective and safe technology and it plays an important role in the remediation of environmental pollutants. In this study, *Pseudomonas fluorescens* ATCC 948 and *Pseudomonas putida* ATCC 12842 were employed to biodegrade phenol, with different culture conditions such as, different incubation periods, initial phenol concentration, temperature, pH, carbon and nitrogen sources) used to examine their effect on phenol degradation. The results showed that phenol degraded completely after 122 h. Different initial concentrations of phenol were added to minimal salts medium and the percentage of phenol degradation decreased as the phenol concentration increased. The optimum temperature for both bacterial strains, *P. fluorescens* and *P. putida,* is 35°C, with the maximum percentage of degradation occuring at pH 7. Glucose is the best carbon source as it increased the rate of biodegradation up to 80%. Ammonium nitrate is the best nitrogen source for *P. fluorescens* to degrade phenol, while ammonium chloride is the best source of nitrogen for degradation of phenol by *P. putida*. The study's results suggest that *P. fluorescens* and *P. putida* are capable of phenol degradation, and thus can be used for bioremediation of synthetic waste water containing phenol.

Key words: Physicochemical factors, biodegradation, *Pseudomonas fluorescens*, *Pseudomonas putida.*

INTRODUCTION

Phenol is present in nature and it is produced in industry. It is an organic compound (Paula and Young, 1998). Phenol and its derivatives are among the most frequently found pollutants in rivers, industrial effluents and landfill run-off waters (Ying et al., 2007; Kotresha and Vidyasagar, 2013). Even if phenol is found in low concentrations of 5 to 25 mg/L, it affects the aquatic environments, leading to bitter taste and bad odour in municipal drinking water. The presence of phenol in water creates significant stress on eco-systems, along with a negative effect on aquatic flora and fauna (Cheela et al., 2014; Sreeremya, 2015).

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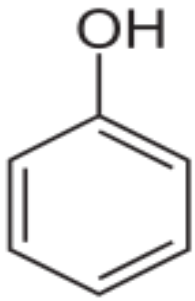
Phenols are used in various industrial applications and industries, such as oil refineries, chemical, petrochemical, pharmaceutical, metallurgical and pesticide products, and in paint and textile industries. Therefore, removal of phenol from industrial aqueous effluents is an important practical problem (Lakshmi et al., 2009). Different types of environmental biotechnologies, such as physical, chemical and biological methods, are used to remove phenol (Mangukiya et al., 2015). Recently developed chemical procedures are specific for phenol removal; these include many expensive methods, such as distillation and liquid-liquid extraction with the use of different solvents, adsorption and membrane filters.

Microorganisms can utilise phenol and its derivatives as a sole carbon and energy source at varying concentrations under optimum conditions (Shweta and Dhandayuthapani, 2013). Biodegradation of phenol occurs in aerobic/anaerobic conditions. Whereas aerobic microbes use oxygen as an electron acceptor, molecular oxygen is a reactant for oxygenase enzymes, and it is incorporated into the final product; in anaerobic conditions, different inorganic electron acceptors are possible, such as NO_3, SO_4, CO_2 and Fe^{3+} (Lakshmi and Sridevi, 2009). Microorganisms and their enzymes can convert phenol to non-toxic intermediates of tricarboxylic acids via meta-pathway (Mohite et al., 2010; Sridevi et al., 2012); however, in higher concentrations, it is difficult to biodegrade phenol and its derivatives because they are toxic to most microorganisms. Phenols can even inhibit the growth of microorganisms that are capable of utilising them. Hence, phenol is used as an antimicrobial agent (Krastanov et al., 2013).

Most types of microbes, including bacteria and fungi, can degrade phenolic compounds and use them as a source of carbon and energy (Michałowicz and Duda, 2007; Supriya and Deva, 2014). Several bacterial species have been studied, including *Acinetobacter calcoaceticus* (Liu et al., 2016), *Pseudomonas sp (*Kafilzadeh et al., 2010), *Bacillus thuringiensis*, *Brevibacterium iodinum* and *Staphylococcus aureus* (Kafilzadeh and Mokhtari, 2013), *Rhodococcus sp*. (Nor Suhaila et al., 2010) and *Nitratireductor aquimarinus, Nitratireductor aquimarius, Marine bacterium* and *Pseudomonas stutzeri* (Boroujeni et al., 2014).

Many studies have recommended using bacteria belonging to the genus of *Pseudomonas* as good degraders of phenol. *Pseudomonas* sp is a Gram–negative bacteria as it is polar flagellated and unicellular. On agar, colonies are circular, have a yellow to greyish colour, 3 to 5 mm diameter, are smooth, glistening and opaque. *Pseudomonas* sp grows well at both 37 and 54°C. No acid or gas is produced from carbohydrates and alcohols, such as arabinose, glucose, fructose, galactose, sucrose, maltose, lactose, starch, inulin, dextrin, glycerol, mannitol and sorbitol. Whereas slight acidity is observed in xylose, nitrate is not reduced, starch is not hydrolysed and gelatin is not liquefied. The catalase, indole and the Methyl Red-Voges Proskauer test results are negative. These organisms do not produce hydrogen sulphide, however, *Pseudomonas* sp grows well in a citrate medium. *Pseudomonas* is aerobic, degrades phenol and resists high concentrations of it (Hamdy et al., 1956; Seker et al., 1997).



Scheme 1. Chemical structure of phenol.

Pseudomonas strains can secret catechol 2, 3-oxygenase, which degrades phenol to catechol (non-toxic intermediate compound). *Pseudomonas putida* can use aromatic compounds such as phenol, as a sole source of carbon and energy. Its optimum microbial growth conditions are 30°C and pH 6.8 (Seker et al., 1997). Also, Hamdy et al. (1956) reported that *Pseudomonas fluorescens* can oxidize phenol to catechol.

Biodegradation is an important process for removing phenolic pollutants. In a biological treatment method it is necessary to select a potential bacterial strain to degrade these pollutants.

Considering the potential of *Pseudomonas* strains, the current study aims to select two bacterial strains, *P. putida* and *P. fluorescens*, and examine their ability to degrade phenol and thereafter, investigate the factors that affect phenol biodegradation.

MATERIALS AND METHODS

Chemicals

Phenol (99% pure, chemical grade), 4-amino antipyrine and all other chemicals were purchased from Merck. Chemical structure of phenol is a white crystalline solid with molecular weight of 94.14 g/mol and formula of C_6H_5OH (Scheme 1) (Sridevi et al., 2012).

Microorganisms

The microorganisms *P. fluorescens* ATCC 948 and *P. putida* ATCC 12842 were obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India and maintained separately on nutrient agar medium containing: Beef extract: 1.0, Yeast extract: 2.0, Peptone: 5.0, NaCl: 5.0 and Agar: 20 in g/L. The pH of the medium was adjusted to 7.0 by adding 1 N NaOH. It was incubated at 37°C for further use.

Biodegradation assay

Degradation of phenol was studied on a phenol supplemented-minimal salts medium (MSM) containing: KH_2PO_4, 2.25; K_2HPO_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 0.1, in g/L; pH= 7. *P. fluorescens* and *P. putida* were inoculated in a 250-ml flask containing 100 ml of MSM separately. The experimental studies were conducted in shake flasks with agitation at a rate of 150 rpm. Bacterial growth was determined in terms of cell mass by measuring optical density at a wavelength of 600 nm using a UV-1800 UV/VIS Spectrophotometer (RAYLEIGH, Beijing Beifen-Ruili Analytical Instrument (Group) Co., Ltd.) (Reshma et al., 2014). The phenol concentrations in medium were determined by the UV spectrophotometer at a wavelength of 272 nm after incubation period and the percentage of phenol removal were calculated using the following equation (Quintana et al., 1997).

$$\text{Percentage of phenol degradation} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

Physicochemical factors

Effect of different incubation periods on the biodegradation of phenol

The efficiency of *P. fluorescens* and *P. putida* to degrade phenol was carried out in 250-ml conical flask containing 100 ml of MSM supplemented with 100 mgl^{-1} phenol and incubated at different periods (24, 48, 72, 96,144 h) under shaking (150 rpm) in a shaking incubator.

Effect of initial concentration of phenol on the biodegradation of phenol

Different initial phenol concentrations (100, 200, 300, 400 and 500 mg/L) were added to the MSM. Flasks were inoculated separately by *P. fluorescens* and *P. putida and* incubated in a shaking incubator (150 rpm) for 96 h. For each concentration of the mixture, three replicates from each treatment were used.

Effect of temperature on the biodegradation of phenol

P. fluorescens and *P. putida* were grown in MS medium separately with (100 mg/L) of phenol at different temperatures (25, 30, 35, 40 and 45°C) and incubated in a shaking incubator (150 rpm) for 96 h. Three replicates from each treatment were used.

Effect of pH on the biodegradation of phenol

The pH of the MSM was adjusted accordingly using 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to pH (6, 7, 8, 9 and 10). Flasks were inoculated separately by *P. fluorescens* and *P. putida and* incubated in a shaking incubator (150 rpm) for 96 h. For every different pH, three replicates from each treatment were used.

Effect of carbon source on the biodegradation of phenol

1 g of different carbon sources such as glucose, sucrose, lactose and fructose were added separately to 250-ml elementary flask containing 100 ml of the MSM to make a 1%w/v solution. Flasks were inoculated separately by *P. fluorescens* and *P. putida and* incubated in a shaking incubator (150 rpm) at 35°C for 96 h. Three replicates from each treatment were used.

Effect of nitrogen source on the biodegradation of phenol

1 g of different nitrogen sources such as urea, ammonium chloride, ammonium nitrate and ammonium sulphate were added separately to 250-ml elementary flask containing 100 ml of the MSM to make a 1%w/v solution. Flasks were inoculated separately by *P. fluorescens* and *P. putida and* incubated in a shaking incubator (150 rpm) at 35°C for 96 h. Three replicates from each treatment were used.

RESULTS AND DISCUSSION

Effect of different incubation periods on the biodegradation of phenol

In the current study, physical and chemical factors were tested to study their effects on biodegradation of phenol. Effect of incubation periods on phenol degradation showed that *P. fluorescens* and *P. putida* are capable of degradation of phenol (Figure 1). Bacterial growth (biomass) increases and phenol degraded completely after 122 h.

Effect of initial concentration of phenol on the biodegradation of phenol

The effect of initial concentration (100 to 500 mg /L) on the biodegradation was studied. Figure 2 showed the ability of *P. fluorescens* and *P. putida* in degradation of phenol. The percentage of phenol degradation decreasing according to increase in phenol concentration (Lakshmi et al., 2009) found cultures inoculated with 0.5 mg/L glucose showed the highest rate of phenol degradation, while the cultures inoculated with the higher concentrations showed a decrease in phenol consumption. In Cheela et al. (2014), the substrate with initial concentration (100 mg/L) was degraded in 96 and 60 h by mixed and pure cultures with a lag phase of 12 and 18 h and suggested that increase in the phenol concentration leads to increase in the degradation time and lag phase. Mohite et al. (2010) noticed the decrease in phenol concentration accompanied with increase in biomass. Moghadam et al. (2016) reported that *Rhodococcus pyridinivorans* degraded 250, 500 and 750 mg/L phenol completely in 24 h. Meanwhile, Ying et al. (2007) reported that high concentration of phenol caused the inhibitory effects on microbial growth. Also, Movahedyan et al. (2009) found that the best phenol-degrading bacteria that completely utilized 500 to 600 mg/L phenol after 48 h incubation belong to *P. putida* strains.

Effect of temperature on the biodegradation of phenol

Figure 3 showed that percentage of degradation is affected by temperature degrees. Optimum temperature

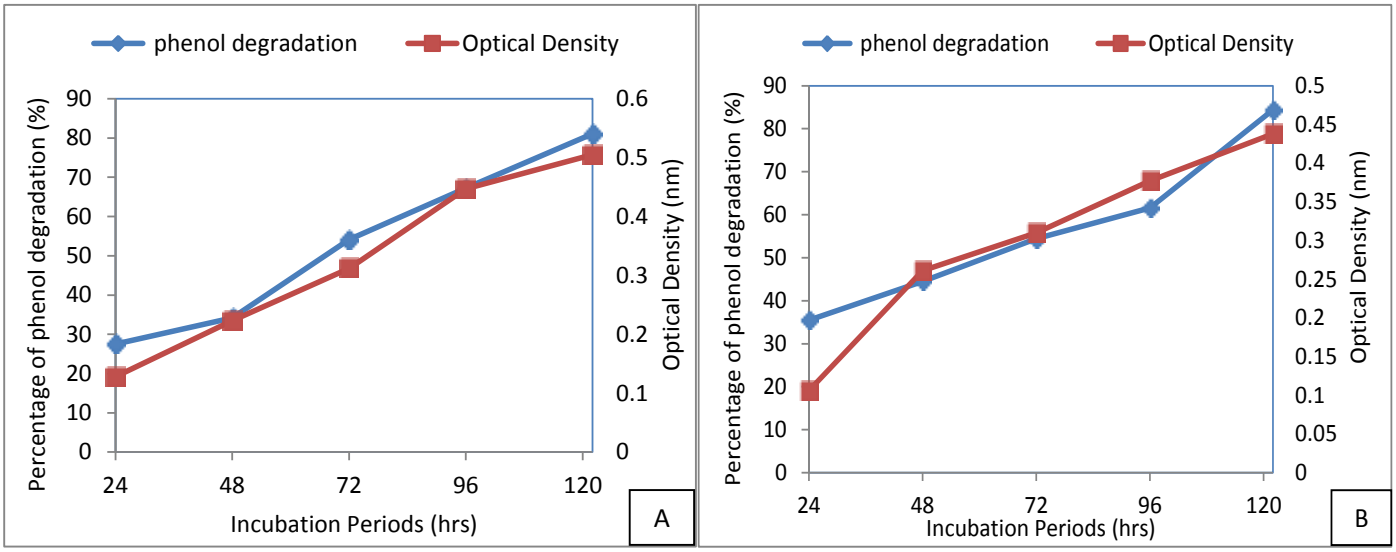


Figure 1. Effect of incubation periods on phenol degradation and growth profiles of the strains (A) *P. fluorescens* and (B) *P. putida.*

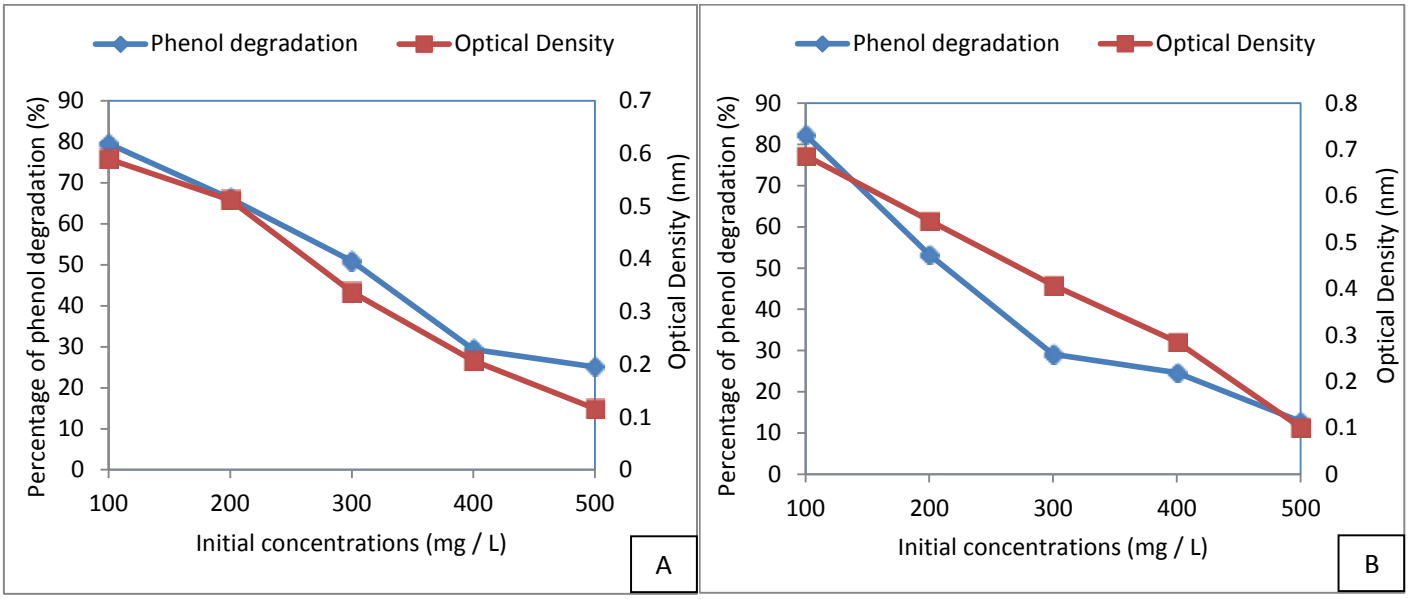


Figure 2. Effect of initial concentrations of phenols on degradation and growth profiles of the strains (A) *P. fluorescens* and (B) *P. putida*.

is 35°C for both *P. fluorescens* and *P. putida.* High temperature degrees (40 to 45°C) led to decrease in the percentage of degradation. Lakshmi et al. (2009) found that *P. aeruginosa* and *P. desmolyticum* degraded phenol rapidly at temperature of 32°C after 24 h of incubation period. Meanwhile, Mohn annd Stewart (2000) reported that temperature plays an important role in affecting petroleum hydrocarbons biodegradation, among the other environmental variables. Shweta and Dhandayuthapani (2013) reported that *P. putida* showed maximum degradation at temperature of 35°C.

Effect of pH on the biodegradation of phenol

Here, the effects of pH values ranging from 6 to 10 were investigated. Figure 4 showed that neutral pH is the best (pH 7). While *P. fluorescens* and *P. putida* recorded a maximum degradation at pH 7, those subjected to extreme alkaline pH (9 to 10) showed a very low percentage of degradation phenol. Rajani and Vijayan (2015) mentioned that when pH decreases, growth also slightly decreases and the degradation does not occur properly. In the case of increasing pH, growth was also

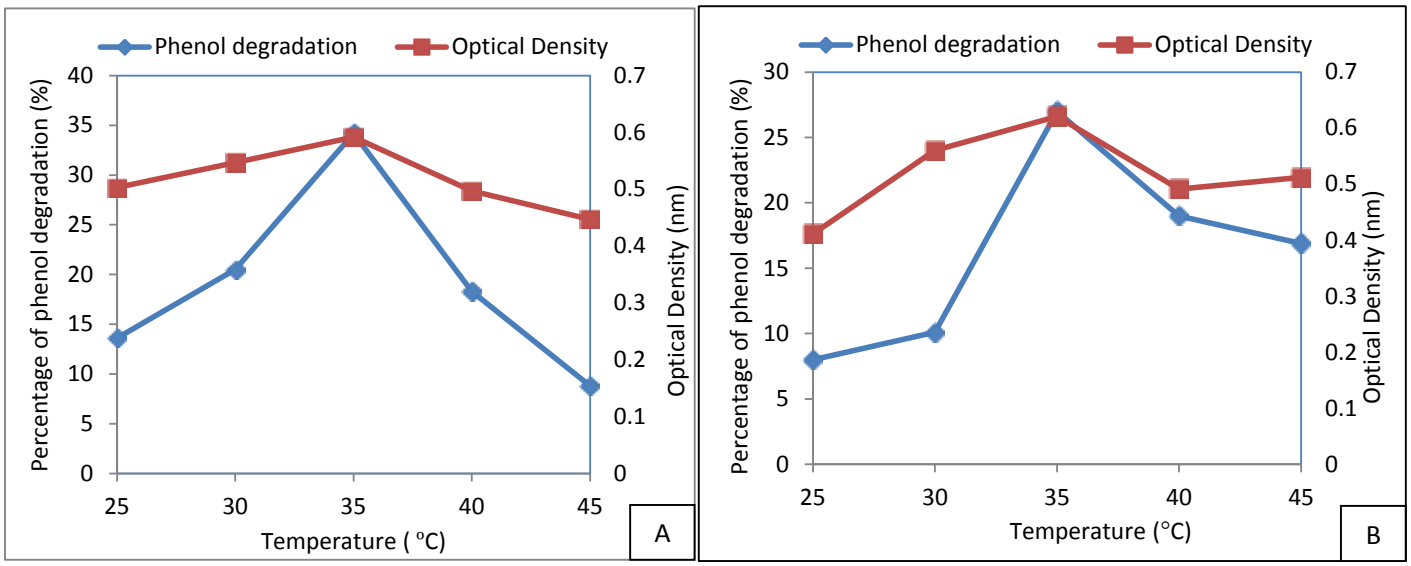


Figure 3. Effect of different temperature degrees on phenol degradation and growth profiles of the strains (A) *P. fluorescens* and (B) *P. putida*.

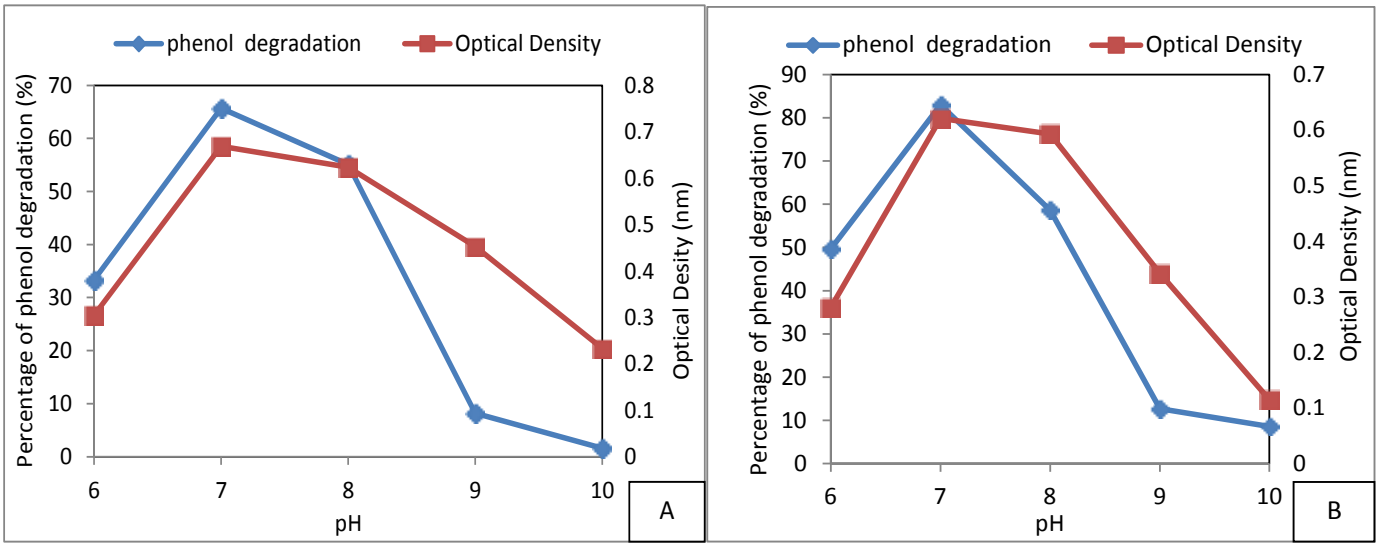


Figure 4. Effect of pH on phenol degradation and growth profiles of the strains (A) *P. fluorescens* and (B) *P. putida.*

increased but degradation rate was very less. Moghadam et al. (2016) found the optimum pH was at 8 for phenol biodegradation by NS1. Mangukiy et al. (2010) found maximum degradation of phenol was at pH 7 by *Candida* spp. Meanwhile, Shweta and Dhandayuthapani (2013) reported that *P. putida* showed maximum degradation at pH 7.

Effect of carbon source on the biodegradation of phenol

Various carbon sources such as glucose, sucrose, lactose and fructose were added separately to MSM. Glucose is the best carbon source which increased the rate of biodegradation up to 80%. Figure 5 showed that glucose is the best source in degradation by *P. fluorescens* and *P. putida.* Medium containing lactose and fructose recorded low percentage in degradation of phenol by *P. fluorescens* while medium containing sucrose and fructose recorded low percentage in degradation of phenol by *P. putida.* Lakshmi et al. (2009) found that the presence of glucose in the culture medium increased the tolerance of the organisms to high phenol concentrations by providing a good source of ready metabolisable carbon to support cell growth.

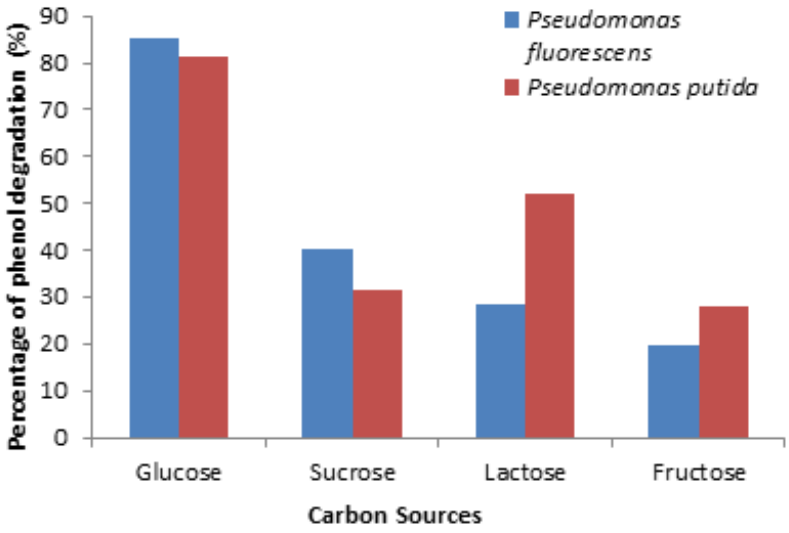


Figure 5. Effect of carbon sources on phenol degradation by *P. fluorescens* and *P. putida.*

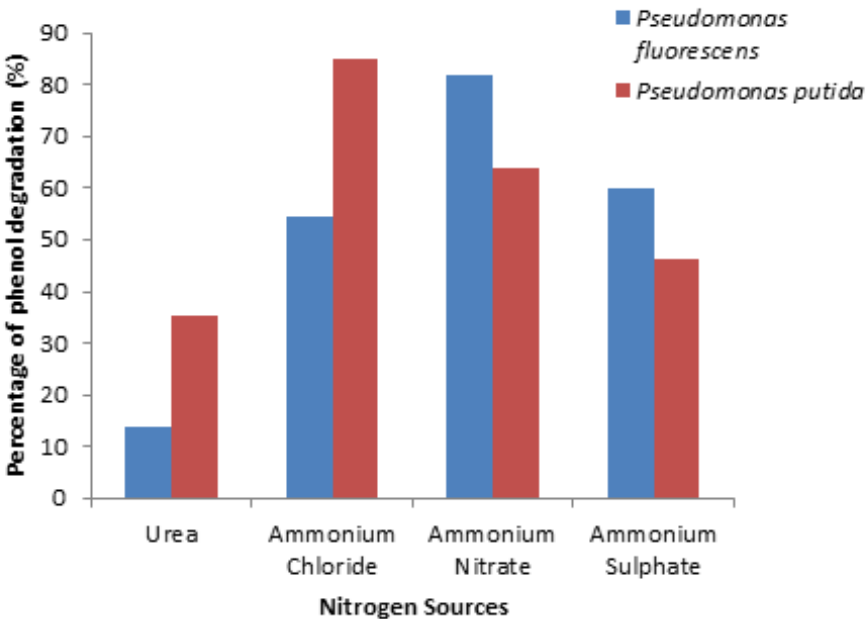


Figure 6. Effect of nitrogen sources on phenol degradation by *P. fluorescens* and *P. putida.*

Effect of nitrogen source on the biodegradation of phenol

Nitrogen sources were tested to examine their ability in degradation of phenol. Figure 6 showed that ammonium nitrate is the best source of nitrogen for *P. fluorescens* to degrade phenol, while ammonium chloride is the best source to degrade phenol by *P. putida.* Medium containing urea recorded the lowest percentage in degradation of phenol. This result agreed with Moghadam et al. (2016) who found that urea showed no significant effects on phenol biodegradation.

Conclusion

This study investigated the biodegradation of phenol using two bacterial strains. It can be concluded that *P.*

fluorescens and *P. putida* have the potential to degrade phenol. Physicochemical parameters can enhance bacterial growth to degrade phenol. The tested strains can remove phenol at optimum temperature 35°C and pH 7 at different initial concentrations. Glucose is the best carbon source as it increased the rate of biodegradation up to 80%. Ammonium nitrate is the best source of nitrogen for *P. fluorescens* to degrade phenol, while ammonium chloride is the best nitrogen source to enhance phenol degradation by *P. putida*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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