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Research Article

STUDY OF PHENOL DEGRADING BACTERIUM ISOLATED FROM A PETROCHEMICAL CONTAMINATED SITE

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ABSTRACT. Phenolic compounds have increasingly found their way into the environment following the industrial revolution. The decontamination of industrial effluents is a prerequisite to effluent treatment and discharge to prevent the detrimental effects of toxic compounds on the environment. In the present study, an attempt has been made to isolate a phenol degrading bacterium and characterize the physicochemical parameters to optimize its degradation potential. To increase the probability of isolating a phenol degrader, the samples were collected from petrochemical sites and the efficiency of bacterium was estimated by 4-amino-antipyrene method. Among the twenty six isolates obtained in our study, *Rhodococcus biphenylivorans (R. biphenylivorans)* strain RARA1707 (NCBI accession no. MK841038) tolerated up to 1100 ppm phenol. Moreover, this isolate could utilize phenol as a sole source of carbon. The optimum conditions for phenol degradation were optimized by 'one factor at a time' approach. *R. biphenylivorans* showed maximum degradation in MSM-D medium (pH: 8) containing 0.45% tryptone, 30°C under shaker condition (130 rpm). The optimum inoculum size was found to be 2% at 0.7 $O.D_{540nm}$. Our study suggests that *R. biphenylivorans* RARA1707 strain is naturally adapted to metabolize phenolic compounds and hence may prove to be a potential candidate for its bioremediation.

Keywords: *4-aminoantipyrene, bioremediation industrial effluent, petrochemicals, Rhodococcus biphenylivorans, toxic compounds.*

INTRODUCTION

The leading causes of environmental pollution are rapid industrialization, agricultural revolution and urbanization. With the ever increasing volume of industrial effluents, the choice of treatment methods to meet the criteria of regulatory bodies, for its discharge, poses a significant challenge. Over 75% of effluent volumes are composed of toxic organic/ inorganic contaminants and dyeing compounds [1]. These effluents severely harm the natural habitat by dispersing through the soil and water bodies. Unfortunately, most of these compounds have formed an irreplaceable part of our day to day lives. For instance, phenols are used widely as disinfectants and antiseptics. They also find extensive application in the form of chlorinated phenols in petrochemical, pharmaceutical, refineries, varnish, pesticide, herbicide, ceramic and steel industries [2, 3].

Phenol is among the highly hazardous chemicals listed by US Environmental Protection Agency (EPA), and is a major pollutant in industrial effluents [4]. The World Health Organization (WHO) has indicated a maximum allowable limit of 1 mg/L phenol in drinking water [5]. It is highly corrosive making it toxic to humans (through skin contact and ingestion) at 10–24 mg/L concentration and fatal at 1 g/kg body weight. Long term exposure can cause anorexia, progressive weight loss, diarrhea and vertigo. They are equally toxic to aquatic and land fauna. The health hazards of phenol intoxication include damage to the heart, liver and kidney [6, 7]. It is also a reported human carcinogen even at low concentrations [2].

The petrochemical effluents have a high biological oxygen demand (BOD), chemical oxygen demand (COD) and total organic carbon (TOC) with pH values in the range of 7-8. Phenol and its derivatives are present in high concentrations in these effluents. Moreover, they have high concentration of non-biodegradable solids which make conventional chemical and physical treatment methods less effective [8]. Remediation of phenol is done using methods like adsorption; reverse osmosis; coagulation/flocculation or high energy irradiation; electrolysis; reduction; electrochemical treatment and ion-pair extraction. More often these techniques are inadequate and complete remediation of phenol requires the use of advanced oxidation techniques using strong oxidizers like hydrogen peroxide and ozone. Also, the conventional methods produce large amounts of sludge which, in turn, requires the use of chemicals for its effective management [9]. Thus, the biological process of effluent treatment using a microorganism capable of utilizing phenol as a carbon and energy source can prove to be an ideal solution to this problem. Such sustainable approaches to effluent treatment are a prominently growing research area today [10]. However, significant insights are still required in the area of biological treatment processes to overcome the drawbacks of conventional methods efficiently and cost effectively. Also, the efficiency of microbial metabolism depends on various physicochemical factors like pH, temperature, aeration, inoculum size and concentration of pollutants. Hence, optimal characterization of these factors can significantly help in enhancing the potential of biological treatment process.

Encouraging findings are reported by Gracioso et al. [11] using *Achromobacter* spp. C-1 for bioaugmentation of phenolic wastewaters. *Pseudomonas* spp. strain NBM11 could also degrade phenolic compounds over a wide pH range [12]. *Nocardia hydrocarbonoxidans* tolerated up to 1300 ppm of phenol [13]. Complete degradation of 2000 ppm phenol was reported by *Rhodococcus pyridinivorans* under optimized conditions in 96h [14]. Similarly, algae, yeast and fungi have also been reported to metabolize phenolic compounds [15, 16]. Considering the above mentioned factors and reported findings, the present study was designed to screen for phenol degraders and determine the optimal performance conditions leading to high biodegradation efficiency.

MATERIALS AND METHODS

Sample collection

The soil samples were collected from petrol pumps (10 samples) and tanneries (5 samples) situated in Mumbai for screening of phenol degrading bacteria. Soil samples were collected in sterile plastic bags, transported to the laboratory and processed immediately.

Enrichment, screening and isolation of phenol degraders

The soil sample (1 g) was suspended in 10 mL of sterile phosphate buffer (pH 7.2), mixed thoroughly and allowed to stand for 30 mins. The enrichment of phenol degraders was done by inoculating 1 mL of the above suspension in 50 mL of Bushnell Hass (BH) broth medium containing 200 µg/mL phenol. The enrichment broth was incubated at 30 °C on shaker (130 rpm) for 7 days [17]. An inoculum size of 2% adjusted to 0.1 $O.D_{540nm}$ was used during the screening process. The screening of phenol degraders was done by isolating enriched samples on BH agar plate containing 200 µg/mL phenol. The plates were incubated at 30°C for 48 h to observe for isolated colonies of phenol degrading bacteria. Individual colonies were re-isolated on BH agar plate containing 200 µg/mL phenol to ensure purity of culture and a single isolated colony was maintained on nutrient agar slants for regular use. The cultures were also preserved on nutrient agar containing 1% glycerol and 500 µg/mL phenol at 4 ℃.

4-Amino antipyrine assay for estimation of phenol

The isolates screened in our study were inoculated in different flasks containing BH broth with 250 to 1500 µg/mL phenol. This was done to selectively screen for highly tolerant strains of phenol degraders. The amount of phenol remaining in the flasks after incubation (until visible turbidity) was detected using the 4-amino antipyrine (4-AAP) assay. The 4-AAP assay is a simple and most sensitive method used for detection of phenols [18]. In this assay, 4-AAP was allowed to react with phenol to give a colored dye compound. This compound was estimated using a spectrophotometer (UV-Vis spectrophotometer 117, Systronics, India) at 540nm. The concentration of phenol was calculated using a standard graph of absorbance (540nm) v/s concentration of phenol (0-60 µg/mL).

Identification of phenol degrading bacteria

The potential isolate showing maximum degradation of phenol was identified preliminarily by morphological, cultural and biochemical tests. The strain was confirmed by 16s rRNA gene sequence analysis. PCR based 16S rRNA gene amplification and sequencing of the isolated bacterium was carried out using universal primers at Sai Biosystems Private Limited, India.

Optimization of parameters to enhance phenol degradation

Phenol is metabolized into simple compounds by phenol degrading bacteria. Hence, this process is greatly influenced by physicochemical parameters such as pH, incubation temperature, incubation time, agitation and inoculum size. The optimization of these parameters was done by applying one factor at a time (OFAT) approach. In this method, one variable of the system is changed at a time while keeping the others constant.

The initial parameters used in our study were BH medium (pH 7), 30℃, shaker conditions (130 rpm) and 2% inoculum adjusted to 0.1 $O.D_{540nm}$. Besides BH medium, 4 mineral medium (MSM-A, MSM-B, MSM-C and MSM-D) were screened for enhancing phenol degradation. The composition of these media is indicated in Table 1. The varying physicochemical parameters optimised in our study included the optical density of test isolate ($O.D_{540nm}$ 0.1-0.7), inoculum size (1%-5%), temperature (4 ℃, 30 ℃, 37 ℃, 55 ℃), pH (4-9) and aeration (0-150rpm) [13, 19, 20].

The influence of carbon sources like glucose, fructose, sucrose, lactose, maltose, mannitol and starch were studied on phenol degradation. Similarly, the effect of organic nitrogen sources (peptone, yeast extract, tryptone and urea) and inorganic nitrogen sources (ammonium sulphate, ammonium chloride, ammonium nitrate, sodium nitrate, and potassium nitrate) on phenol degradation was also studied. In addition, the effect of increasing concentration of suitable nutrient sources on phenol degradation was observed [21, 22].

Table 1. *Composition of media screened for enhancing phenol degradation*

Sr. No.	Media	Composition (in g/L)
1	MSM-A	K_2HPO_4 (0.4), KH_2PO_4 (0.2), NaCl (0.1), $MgSO_4.7H_2O$ (0.1), $MnSO_4.H_2O$ (0.01), $Fe(SO_4).H_2O$ (0.01), $NaMoO_4.2H_2O$ (0.01), $(NH_4)_2SO_4$ (0.4), pH 7
2	MSM-B	K_2HPO_4 (1.5), KH_2PO_4 (0.5), $(NH_4)_2SO_4$ (0.5), NaCl (0.5), Na2SO_4 (3.0), Yeast extract (2.0), $Fe(SO_4).H_2O$ (0.002), $CaCl_2.2H_2O$ (0.5), pH 7
3	MSM-C	K_2HPO_4 (0.42), KH_2PO_4 (0.37), $(NH_4)_2SO_4$ (0.24), NaCl (0.015), $CaCl_2.2H_2O$ (0.015), $MgSO_4.7H_2O$ (0.05), $FeCl_3.6H_2O$ (0.054), pH 7
4	MSM-D	$(NH_4)_2SO_4$ (3.0), KH_2PO_4 (1.0), $MgSO_4.7H_2O$ (0.2), $FeCl_3.7H_2O$ (0.02), pH 7
5	BH	$MgSO_4.7H_2O$ (0.2), $CaCl_2.2H_2O$ (0.02), K_2HPO_4 (1.0), KH_2PO_4 (1.0), $(NH_4)_2NO_3$ (1.0), $FeCl_3.6H_2O$ (0.05), pH 7

Statistics

All of the experiments were carried out in triplicate, and the mean values with standard deviation were reported.

RESULTS AND DISCUSSION

Screening of potential phenol degrading bacteria

On screening, 26 phenol degrading isolates were obtained in our study. These isolates were further tested for phenol tolerance. The amount of phenol degraded by all isolates screened in our study is indicated in Fig. 1. Among these isolates, PD 17 tolerated 1100 µg/mL phenol and was selected for further studies. It showed 72.74 % degradation of 1100 µg/mL phenol (Fig. 2) in BH medium (pH 7) at 30 °C under shaker condition (130rpm) in 96 h when 2 % (0.1 $O.D_{540nm}$) inoculum was used.

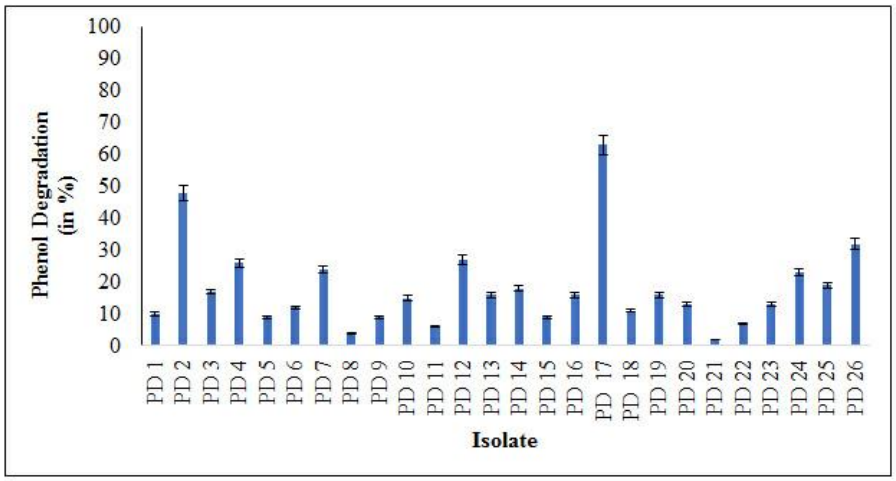

Fig. 1. *Screening of potential phenol degrading isolates*

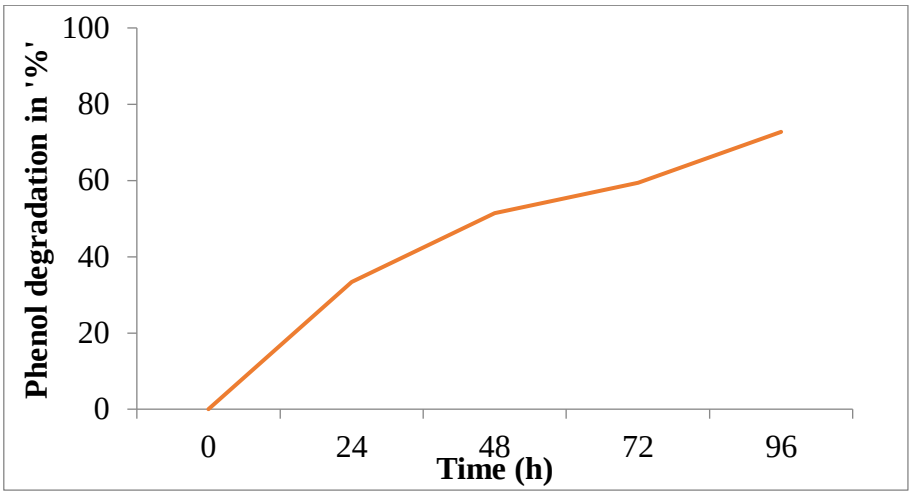

Fig. 2. *Degradation of phenol in BH medium by PD-17 in 96h*

The natural adaptation of micro-organisms to polluted environments is an interesting phenomenon commonly observed in nature. Soil samples contaminated with hydrocarbons suitably increase the probability of isolation of phenol degraders. Hence, petrol pumps and tanning industry (where phenols are used for tanning and dyeing of leather) sites were selected for collection of samples in our study. An early study from Canada described the phenol contaminated status of St. Lawrence River [23]. It was among the first studies to evaluate the phenol tolerance of the river microflora. They reported that over 70% microflora in the polluted part of the river could tolerate around 150 ppm phenol. This was 40% more than the tolerant microflora in the unpolluted part of the river. The study further suggested the seasonal fluctuations in the microflora and their phenol degradation potential, due to changes in temperature. Following this study, several potential phenol degrading bacteria has been isolated from phenol contaminated sites like coke processing wastewater, petrochemical effluents, pharmaceutical wastes, landfill sites and even activated sludge [10, 24-28].

Identification of promising strain by 16s rRNA

PD-17 was identified as gram positive, and pleomorphic isolate that degraded only sucrose among the tested sugars under anaerobic conditions. The colony and biochemical characteristics of PD-17 are represented in Table 2 and 3 respectively. It was tolerant to 6.5% NaCl and showed a positive ornithine, lysine, nitratase and VP test. On 16s rRNA analysis it was identified as *Rhodococcus biphenylivorans*. The phylogram of this strain is represented in Figure 3. The gene sequence of the isolate was submitted to NCBI (accession no. MK841038) and the strain was named as RARA1707. The *Rhodococcus* sp. belongs to the family Nocardiaceae and suborder Corynebacterineae [29]. The members of the genus *Rhodococcus* show vast diversity in their cultural and biochemical characteristics [30, 31]. Significant potential of these organisms in bioremediation of organic recalcitrant pollutants can be clearly observed from literature survey [32-34]. However, very few studies have described the potential of *Rhodococcus* sp. to degrade phenolic compounds including 2, 4-dinitrophenol, nitrophenol and polychlorinated biphenyl [35-38].

Table 2. *Colony characteristics of Rhodococcus biphenylivorans strain RARA1707*

Colony characteristics	**Size**	**Shape**	**Color**	**Margin**	**Opacity**	**Consistency**	**Gram nature and morphology**
Observation	2mm	circular	peach	entire	concave	butyrous	Gram positive pleomorphic

Table 3. *Biochemical characteristics of Rhodococcus biphenylivorans strain RARA1707*

Test	**Observations**	
Sugar fermentation	**Aerobic**	**Anaerobic**
Glucose	-	-
Xylose	-	-
Galactose	-	-
Mannose	-	-
Mannitol	-	-
Xylulose	-	-
Sucrose	-	+
Fructose	-	-
Inulin	-	-
Maltose	-	-

Table 3. *(Continues)*

Test	**Observations**	
Sugar fermentation	**Aerobic**	**Anaerobic**
Inoline	-	-
Arabinose	-	-
Ribose	-	-
Trehalose	-	-
Raffinose	-	-
Sorbitol	-	-
Lactose	-	-
Biochemical Test		
Ornithine	+	
Lysine	+	
Nitratase	+	
Gelatinase	-	
Methyl red	-	
VP test	+	
Starch hydrolysis	-	
Bile esculin	-	
Growth in 6.5% NaCl	+	

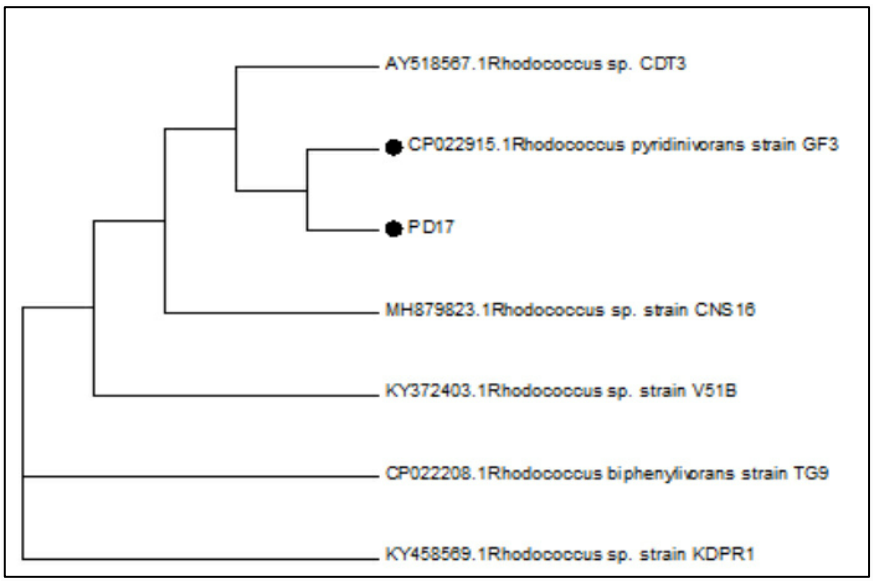

Fig. 3. *The phylogram of Rhodococcus biphenylivorans RARA1707*

Optimization of physicochemical parameters to enhance phenol degradation

The aerobic biodegradation of phenol is a preferred method in industrial waste water treatment to prevent bad odour, provided it is completely metabolized or mineralized in the process [39]. As suggested by Israni et al. [40], it is important to study the effect of process variables on the biodegradation of phenol. For an aerobic micro-organism, increasing the temperature, aeration speed and organic loading rate can support microbial activity. Temperature also plays an important role in the maintenance of osmosis, nutrient availability and metabolism [41]. Hence, better performance can be expected by increasing these parameters in terms of phenol removal. On the other hand, increasing the hydraulic loading rates negatively affects phenol degradation [40]. Similarly, an initial high concentration of phenol can cause significant stress on the isolate and hence lead to ineffective phenol removal. Besides these parameters, the pH of the medium and its composition also affects the biodegradation capabilities of the isolate [17, 42].

Among the media used in our study, *R. biphenylivorans* RARA1707 showed maximum degradation in MSM-D medium. The study of the effect of physicochemical parameters revealed optimum degradation at pH 8, 30°C at 130rpm in presence of 2% (at 0.7 $O.D_{540nm}$) inoculum size in 48h (Fig. 4). The initial inoculum used in our study was 2% at 0.1 $O.D_{540nm}$ which degraded 72.74 % phenol in 96 h. The optimum inoculum size showed over 50 % reduction in time (48 h) to degrade 99 % phenol. As observed in Fig. 4, an increase in inoculum size (above 2%) and optical density (above 0.7 $O.D_{540nm}$) did not affect the rate of phenol degradation. This observation is particularly relevant to this study, since it indicates that though *R. biphenylivorans* can utilize phenol as a sole carbon source, its rate of assimilation is not proportional to the inoculum size. In simple words, if this strain is used for bioaugmentation or bioremediation process, it will be easier to utilize higher inoculum size to prevent culture dilution at the environmental sites, without facing the drawback of the strain reaching lag phase sooner. However, *R. biphenylivorans* does show stringent requirements for controlling temperature, pH and media. Hence, it will be more practical to use this strain under isolated laboratory or environmental conditions for remediation of small sites at a time.

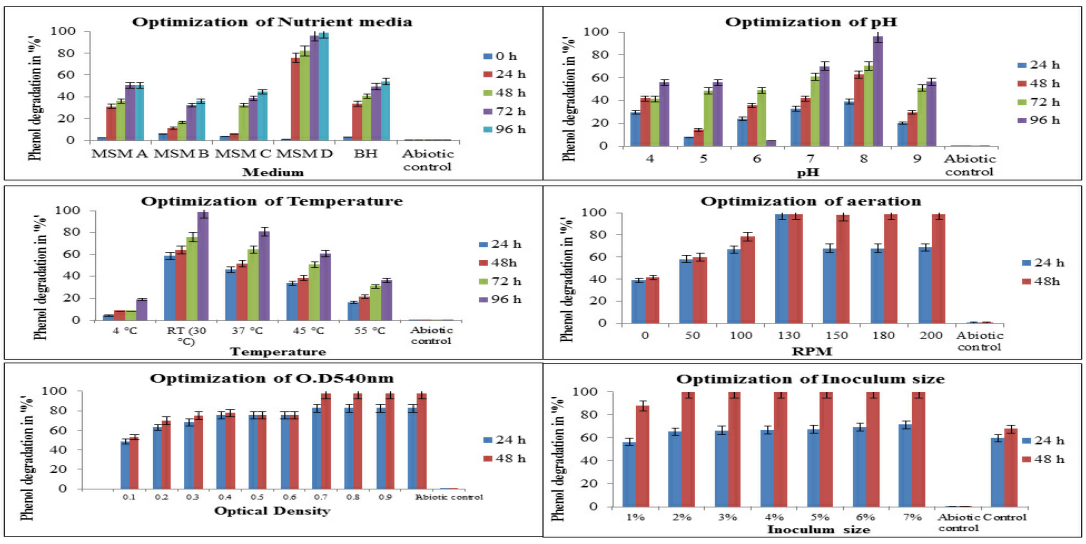

Fig. 4. *Optimization of physicochemical parameters to enhance phenol degradation by R. biphenylivorans RARA1707*

Among the phenol degraders mentioned in the literature, *Acinetobacter* spp. and *Alcaligenes* spp. have shown considerable potential. *Acinetobacter calcoaceticus* isolated from petroleum wastewater degraded 91.6% of 800 mg/L phenol in 48h and tolerated up to 1700 mg/L phenol [43]. Another *Acinetobacter* spp. strain PD12 metabolized 99.6% of 500 mg/L phenol within 9 h and tolerated up to 1100 mg/L phenol [44]. *Alcaligenes faecalis*, isolated from activated sludge degraded 1600 mg/ L phenol in 72h [45]. However, very few studies have reported high phenol tolerance in a *Rhodococcus* spp. For example, *R. aetherivorans* UCM Ac-603 could metabolize 1750 mg/L phenol in 96 h by using it as a sole carbon source [46]. Comparing the concentration of phenol tolerated by the above strains, the observations in our study may not seem as challenging. However, based on the observations of the inability of the isolate to metabolize most sugars (Table 3), it can be suggested that *R. biphenylivorans* RARA1707 is naturally adapted to survive in phenol contaminated sites. Hence, it may be easier to increase the tolerance of this strain by gradually exposing it to increasing concentrations of phenol. Besides, the time factor is critical for practical application of any process, and hence *R. biphenylivorans* RARA1707 may potentially challenge other strains in bioremediation of phenol.

Although optimum degradation was observed at pH 8 and 30 °C in our study, *R. biphenylivorans* RARA1707 showed considerable activity up to 45 °C and between pH 7-9. Also, unlike other reported studies, the increase in speed of aeration (up to 200 rpm) did not affect its degradation potential. Similarly, phenol degrading *Rhodococcus* strain AQ5NOL2 showed temperature optima in the range of 20 °C and 35 °C [47]. *Pseudomonas aeruginosa* strain MTCC 4996 tolerated 15°C–45 °C temperature and pH range from 6-10 with an optimum of 37 °C and pH 7. It degraded 1200 mg/L and 1300 mg/L phenol in 132h and 156h respectively under shaker conditions. In contrast to our observations, the increase in aeration reduced the phenol degradation efficiency of *P. aeuginosa* MTCC 4996 from an initial 66h to 108h [48].

The temperature and pH optima of the phenol degraders largely depend on their nature and environment from where they are isolated. This is because their metabolic process is naturally adapted to function in a suitable environment. Hence most studies in which the isolation is done from soil sample show optimum degradation at 30 °C and near neutral pH (6-8). Few examples include the degradation of phenol by *Rhodococcus pyridinivoran* (pH 8), *Pseudomonas resinovorans* strain P-1 (pH 7), and *Pseudomonas fluorescens* (pH 7) [12, 14, 49]. Similarly, an Antarctic halophilic bacterium *Rhodococcus* sp. strain AQ5-14 degraded 500 mg/L phenol optimally at pH 7 and 25 °C in a medium containing 0.4 g/L NaCl [50]. A cold tolerant *Arthrobacter* spp. AG31 and mesophilic *Pseudomonas putida* DSM6414 strains could degrade only 200 mg/L (in 48h) and 400 mg/L (72h) phenol at 10°C and 25 °C respectively [51].

Effect of additional carbon and nitrogen sources on phenol degradation

In the present study, the addition of carbon sources showed a negative effect on phenol degradation (Fig. 5). In contrast, the addition of 0.45% tryptone enhanced phenol degradation and also reduced the time from 48h to 24h (Fig. 5). Further increase in the concentration of tryptone led to considerable increase in the pH of the medium which affected the growth of the isolate leading to reduced degradation of phenol.

A vast diversity in biochemical characteristics of *Rhodococcus* sp. has been reported in literature [30]. However, to the best of our knowledge, this is the first study reporting the ability of aerobic *R. biphenylivorans* RARA1707 isolated from natural environment to metabolize phenol selectively as opposed to any other common carbon sources. This

unusual characteristic may be a key to discovering novel enzymes or metabolic pathways for assimilation of phenol. In addition, the ability to utilize phenol as a sole carbon source favours the cost effective bioremediation approach.

In general, biological treatment methods are associated with certain environmental risk factors like mutations and negative association of test strains with other species in an ecosystem. However, the probability of these observations is more likely in engineered strains. The natural isolates, on the other hand are more stable. Since *R. biphenylivorans* RARA1707Ideally, an easily metabolized carbon source favours phenol degradation by acting as an energy source to increase the density of the micro-organism, which, in turn, assimilates phenolic compounds. For instance, SP-4 and SP-8 tolerated 1600 mg/L and 1800 mg/L phenol in presence of 1% glucose in minimal salt medium, in a study carried out by Sachan et al. [52]. These isolates failed to grow in absence of glucose. Moreover, the carbon sources in the medium may cause catabolic repression and prevent optimum assimilation of phenolic compounds. The catabolic repression of phenol by glucose is well demonstrated by Papanastasiou and Maier [53] and Satsangee and Ghosh [54]. The nitrogen sources like peptone and tryptone have been reported to attenuate phenol toxicity leading to increased microbial cell mass [55. 56]. Hence, as observed in this study, suitable concentration of nitrogen sources has significant impact on the phenol degradation. All these factors collectively favour the potential of *R. biphenylivorans* RARA1707 in bioremediation of phenol. In contrast to this study, phenol degrading *Rhodococcus* strain AQ5NOL2 showed best phenol degrading potential in presence of ammonium sulphate, glycine or phenylalanine [47]. Another strain of *R. pyridinivorans* showed optimal biodegradation at pH 8 and 30 °C, in presence of urea (1g/L) and K_2HPO_4 (0.5g/L) [20].

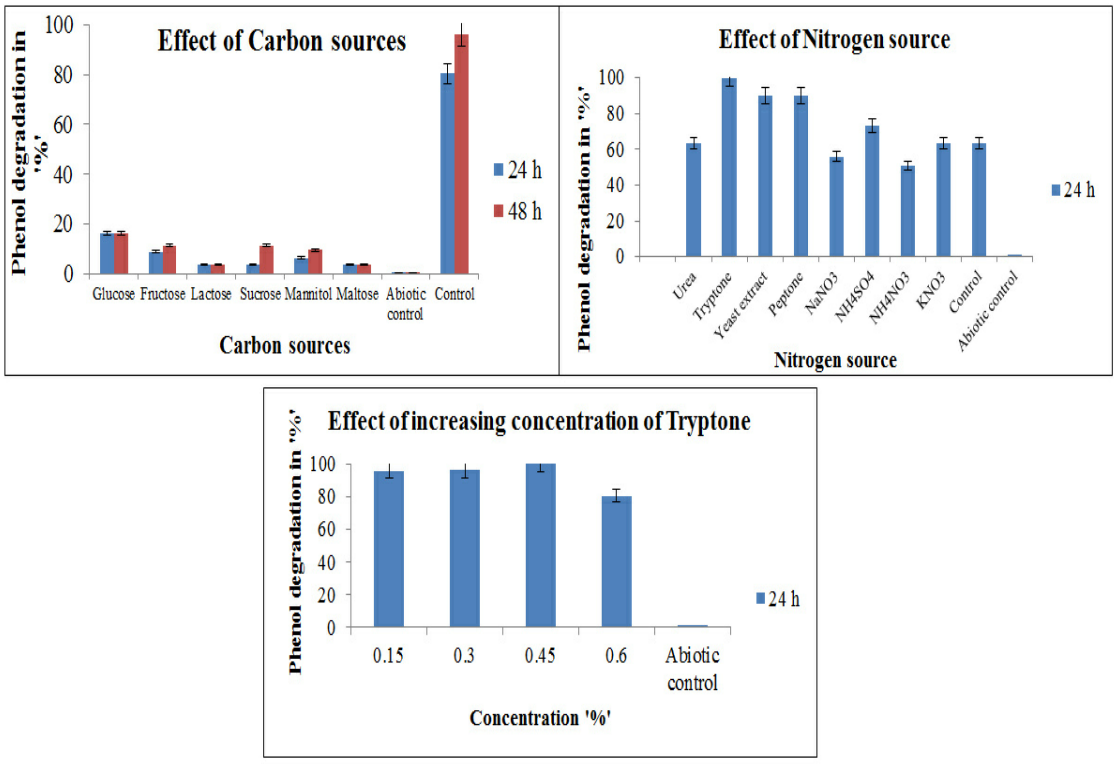

Fig. 5. *Effect of carbon and nitrogen sources on phenol degradation by Rhodococcus biphenylivorans RARA1707*

CONCLUSION

The sustainable approach of biodegradation of xenobiotic compounds is the most promising strategy in the detoxification of polluted sites. The ability of *R. biphenylivorans* to metabolize phenol selectively makes it the best candidate for its bioremediation. Although it can be gradually adapted to grow in higher phenol concentration and immobilized to further improve its efficiency. Immobilized cells can be protected from inhibitory effect of various contaminants present in industrial effluents. Also the cells can be recycled or used for continuous set-up for bioremediation of phenol. The optimization of simple physicochemical parameters in this study enhanced phenol degradation in just 24h, from an initial 96h. This suggests immense value of *R. biphenylivorans* RARA1707 in bioremediation of phenol. To thoroughly exploit the full potential of this strain, further analytical studies may help tremendously by analysing the biochemical pathway of phenol degradation. Even if the time period required for phenol degradation is not reduced; simple steps in further studies will ensure considerable strain improvement.

Conflict of Interest. The author declared that there is no conflict of interest.

Authorship Contributions. Concept: A.S.P., Design: A.S.P., Data Collection or Processing: A.S.P., A.H.P., R.M.M., R R.K., Analysis or Interpretation: A.S.P., Literature Search: A.S.P., A.H.P., R.M.M., R R.K., Writing: A.S.P

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