

## Viability, survivability and tolerability of *Rhodococcus pyridinivorans* GM3 for phenol concentrations

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### To cite this article:

Al-Defiery, M. E. and Gopal, R. Viability, Survivability and Tolerability of *Rhodococcus pyridinivorans* GM3 for phenol concentrations, *Mesop. environ. j.*, 2015, Vol. 1, No.2, pp. 57-65.

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### Abstract

Microorganisms play a major role in saving our environment by degrading xenobiotic compounds and chemical wastes, which are toxic either in their native or modified form. Bacteria capable of degrading phenol are common and comprise aerobic and anaerobic catabolizing phenol as a sole source of energy and carbon.

The bacterial isolate *Rhodococcus pyridinivorans* GM3 was studied for viability, survivability and tolerability GM3 for different concentrations of phenol in mineral salts medium (MSM). The results showed that *R. pyridinivorans* GM3 had powerful adaptability to the some conditions of environment, which exhibited that it has a high survivability and tolerability upto 7.0 g/L of phenol concentration in the medium. The tolerance of microorganism to phenol toxicity reduced with increasing concentrations. There was increase in survivability of *R. pyridinivorans* GM3 on MSM when inoculum size was increased from 1% to 4%. The results suggest that inoculum size led to increase survivability and tolerability. The results of this study clearly demonstrate that presence of phenol in media upto 2.0 g/L does not adversely affect the viability. However, the inhibitory effect of phenol on viability indicated that GM3 could not degrade 2.5 g/L phenol concentration with 1% inoculum size. *R. pyridinivorans* GM3 exhibited highest potential for phenol concentrations.

**Keywords;** Phenol, *Rhodococcus*, Viability, Survivability, Tolerability

### Introduction

A wide variety of synthetic chemicals has found their way into the ecosystem. Among the different toxic compounds, phenol is recognized as a pollutant and phenol contaminated water is a potential threat to human health because it is hematotoxic and hepatotoxic, provoke mutagenesis toward humans and other living organisms [1]. Phenol and its derivatives are some of the major hazardous compounds in industrial wastewater produced from various industrial activities. This extra accumulation of phenol in nature leads to

change in microbial biodiversity and has abusive trail on human health. Phenol is an aromatic molecule containing hydroxyl group attached to the benzene ring with the chemical formula  $C_6H_5OH$ .

Phenol is a powerful microbicidal substance, which is obtained by distillation of coal tar between temperature of 170°C and 270°C. Lister, the father of antiseptic surgery, first introduced them in surgery (1865), since then a wide range of phenolic compounds has been developed as disinfectants [2]. Phenol is toxic to bacteria; therefore, it is also used as an antiseptic, because of its anesthetic properties, phenol is used in medicines such as ointments, nose and ear-drops, throat lozenges and sprays, sore lotions, cold and antiseptic lotions [3]. Phenol is not easily biodegradable and difficult to use as a substrate for growth because it inhibits the innate activity of most types of microorganisms at higher and lower concentrations, and is toxic even at low concentrations and there are toxicity reports on microbial cells [4]. The ingestion of one gram of phenol can have fatal consequences in humans; phenols are careful as toxic for some aquatic forms of life in concentrations higher to 50 ppb and [5].

Microorganisms such as bacteria, fungi, yeast and algae from a variety of genera and species can metabolize this phenol [6, 7, 8, 9]. Patel and Rajkumar[10] reported that the isolate *Saccharomyces cerevisiae* was tolerant to phenol toward 800 mg/L and the phenol degraded was 8.57% at phenol concentration at 800 mg/L. Research is focused to expand the range of microorganisms used for bioremediation and to isolate naturally occurring microbes that have better pollutant degradation capacity. Nevertheless, microorganisms are known to develop mechanisms to survive and resist phenol at concentrations that are normally inhibitory to microbial activity [11]. Therefore, phenol and phenolic compounds are active against vegetative bacteria (Gram-positive and Gram-negative) but practically inactive against spores, they are fungicidal and also kill some viruses [12]. Putriņš *et al.* [13] showed that phenol caused accumulation of cells with larger DNA content indicating cell division arrest. Single cell analysis data designated that the cell division step of cell cycle is particularly sensitive to the toxic phenol effect and its inhibition can be considered as an adaptive response under conditions of phenol stress. Whilst Khleifat[14] observed that, phenol inhibits the growth rate of bacterium *Ewingella americana* with a maximum concentration of 1100 mg/L, beyond which no growth occurred. Moreover, it is necessary information about the factors controlling the metabolism and growth of microorganisms in polluted environments because several of the above criteria are highly empirical rather than knowledge based[15].

Nowadays, *Rhodococcus* is considered as very important organism, especially with their remarkable versatile metabolic power, with the ability to assimilate a wide variety of organic compounds, including hydrophobic xenobiotic. Their general tolerance to toxic substrates, solvents, desiccation and other environmental stress factors, as well as their mycolic acid surfactants produced by these bacteria, which may facilitate uptake of hydrophobic compounds [16]. Genomes analysis genomes of *Rhodococcus* strains is beginning to detect why they are adaptable and have adopted by a hyper recombination evolutionary strategy that relies upon the acquisition and storing of many genes to organize as recombination substrates upon adaptation [17]. Parameters such as concentrations of pollutant, viable biomass, inhibitor and microbial adaptation are the most important parameters that affect phenol biodegradation rate [18]. Bacteria that can tolerate, survive and multiple in the presence of phenol can play a significant role in phenol elimination. Therefore, the objective of study to investigate viability, survivability and tolerability bacterial isolate *Rhodococcus pyridinivorans* GM3 for phenol concentrations.

## Materials and methods

### Isolation

Enrichment medium of phenol degrading bacteria was carried out to screen soils sample, one of the bacterium strain that isolated showed high phenol degradation under aerobic condition and has been identified as *Rhodococcus pyridinivorans* GM3 by microscopic, morphological and biochemical characteristics.

### Growth medium

The mineral salts medium (MSM) consists of (g/L), 1.25 of yeast extract, 0.35 of  $K_2HPO_4$ , 0.35 of  $MgCl_2 \cdot 6H_2O$ , 0.2 of  $Ca(NO_3)_2$ , 0.12 of  $FeCl_2$  and trace elements(0.2 mg/L  $CuSO_4 \cdot 5H_2O$ , 0.1 mg/L  $ZnSO_4 \cdot 7H_2O$ , 0.2 mg/L  $MnSO_4 \cdot 2H_2O$  and 0.1 mg/L  $Na_2MoO_4$ ) with phenol as the sole carbon source.

## **Inoculum preparation**

*R. pyridinivorans* GM3, isolated from soil in lab (Department of Microbiology- Osmania University) by enrichment culturing with phenol. Actively growing culture of *R. pyridinivorans* GM3 was inoculated (loop full) into MSM broth with 1% glucose and 0.05% phenol and incubated at 32°C and with agitation 200 rpm (optimization conditions) for 20 hours (approximately 10<sup>9</sup> CFU/mL).

## **Viability of *R. pyridinivorans* GM3 for phenol**

The *R. pyridinivorans* GM3 was tested for its viability in presence of phenol in MSM containing varying concentrations of phenol (1.0, 1.5, 2.0, 2.5 and 3.0 g/L). 1% (v/v) of inoculum was transferred into a 250 mL conical flask containing 50 mL of MSM containing varying concentrations of phenol (absence of phenol in MSM was used as control for initial inoculum and direct culturing for counting). Triplicates were incubated at 32°C and 200 rpm for 24 hours. Total viable count was enumerated by spread plate technique using 0.1 mL of the dilution 10<sup>1</sup> to 10<sup>9</sup> onto MSM agar. The colony forming units (CFU) 30-300 on each plate were counted using a colony counter. Total number of colonies represents total number of viable cells. The counting of number of colonies in each plate and calculating the titer value for viability, survivability and tolerability test were performed using the following formula:

$$\text{Viable Count} = \text{Number of Colonies in Given Plate} / \text{Dilution Factor} \times \text{Volume Plated}$$

## **Survivability of *R. pyridinivorans* GM3 for phenol**

*R. pyridinivorans* GM3 was tested for its survivability for phenol in triplicate using MSM (50 mL) containing different concentrations of phenol (3.0-8.0 g/L) in 250 mL flasks, and inoculated with different inoculum size ranging from 1 to 4% (v/v) of *R. pyridinivorans* GM3. Incubation was done at 32°C with agitation 200 rpm and pH 8.5 for 24 hours.

Total viable count was enumerated by spread plate technique using 0.1 mL of the dilution 10<sup>1</sup> to 10<sup>6</sup> onto MSM agar. The colony forming units (CFU) 30 -300 on each plate were counted using a colony counter. Total number of colonies represents total number of viable cells.

## **Tolerability of *R. pyridinivorans* GM3 for phenol**

Bacterial isolate *R. pyridinivorans* GM3 was tested for phenol tolerance in triplicate using MSM (50 mL) containing 1% glucose with different concentrations of phenol (3.0-8.0 g/L) in 250 mL flasks, and inoculated with different inoculum size ranging from 1 to 4% (v/v) of *R. pyridinivorans* GM3. Incubation was done at 32°C with agitation 200 rpm and pH 8.5 for 24 hours.

Total viable count was enumerated by spread plate technique using 0.1 mL of the dilution 10<sup>1</sup> to 10<sup>6</sup> onto MSM agar. The colony forming units (CFU) 30-300 on each plate were counted using a colony counter. Total number of colonies represents total number of viable cells.

## **Results**

### **Viability of *R. pyridinivorans* GM3 for phenol**

Viability is defined as an organism's ability to multiply. Viability depends on age, nutrient factors, environmental factors, etc. Higher concentration of any toxic chemical may affect survival or viability of microorganisms. Viability studies of *R. pyridinivorans* GM3 were carried out on MSM (Figure 1) to determine the inhibition effect of various phenol concentrations on the growth within 24 hours. Initial inoculum added was 33×10<sup>6</sup> CFU/mL and it was observed that after 24 hours of incubation, the population of *R. pyridinivorans* GM3 showed progressive increase viability (53×10<sup>8</sup> CFU/mL) at concentration 1.5 g/L

but not at 2.0 g/L of phenol concentration. Subsequently at 2.5 and 3.0 g/L of phenol concentrations with 1% inoculum showed inhibitory effect on the growth of *R. pyridinivorans* GM3 as indicated. The results of this study clearly demonstrate that presence of phenol in media upto 2.0 g/L does not adversely affect the viability

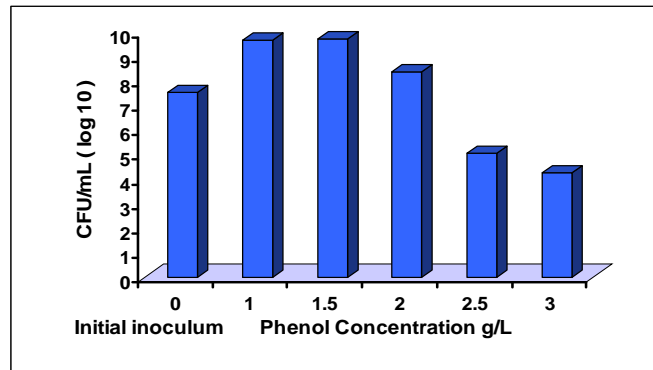


Figure 1. Viability of *R. pyridinivorans* GM3 with different concentrations of phenol

### Survivability of *R. pyridinivorans* GM3 for phenol

The ability of microorganisms to remain alive or continue to exist is known as survivability. Survivability of *R. pyridinivorans* GM3 was studied with exposure at various phenol concentrations (Figure 2). There was an increase in survivability of *R. pyridinivorans* GM3 on MSM when inoculum size was increased from 1% to 4%. However, for different concentrations of phenol (3, 4, 5, 6, 7 and 8 g/L) checked for survivability of *R. pyridinivorans* GM3, it showed that at 8 g/L phenol there was no growth of *R. pyridinivorans* GM3. From this result, it can be concluded that the adaptation of *R. pyridinivorans* GM3 to phenol was necessary for survival and for biodegradation of higher concentration of phenol in a medium.

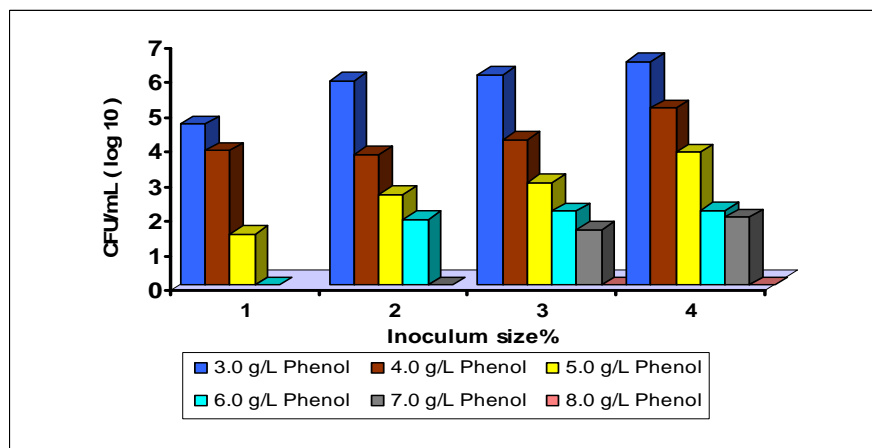


Figure 2. Survivability of *R. pyridinivorans* GM3 with different concentrations of phenol

### Tolerability of *R. pyridinivorans* GM3 for phenol

Tolerability indicates the resistance of microorganisms to the toxic effect of a particular chemical. The tolerance of *R. pyridinivorans* GM3 to phenol in MSM with glucose as an alternative carbon source was investigated. Figure 3 shows the bactericidal effect of phenol on the freely suspended cell cultures at various phenol concentrations. It was observed when phenol concentration was more than 7.0 g/L it

significantly declined the cell number resulting in cell death. The results suggest that inoculum size led to increase survivability and tolerability. Similar pattern was observed for both survivability and tolerability, nevertheless presence of glucose in media was not significant for enhancing *R. pyridinivorans* GM3 to phenol resistance.

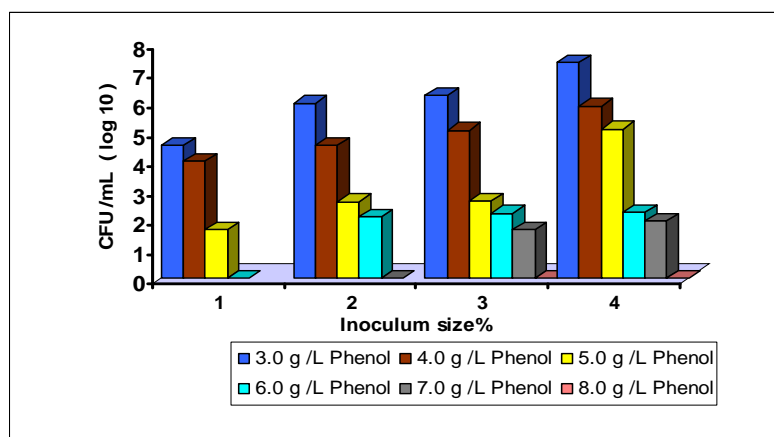


Figure 3. Tolerability of *R. pyridinivorans* GM3 with different concentrations of phenol

## Discussion

Phenol exerts a general bactericidal effect because of the compound's ability to partition into cell membranes, which leads to a loss of cytoplasmic membrane integrity. Phenol toxicity results in disruption of microbial activities associated with energy transformations, membrane barrier functions, and membrane protein functions, which cause eventual cell death.

The results clearly demonstrated that presence of phenol in media does not adversely affect the viability of *R. pyridinivorans* GM3 after 24 hours at 1.0 and 1.5 g/L, while the inhibitory effect of phenol on viability indicated that it cannot degrade phenol at more than 2.0 g/L concentrations with 1% inoculum size (Figure 1). Because of the inhibitory nature of phenol to *R. pyridinivorans* GM3 populations at high concentrations of phenol, an accidental release, may completely inhibit microbial degradation or result in very long lag phase following a lower biodegradation rate. Increasing the initial concentration of phenol from 100 mg/L to 500 mg/L resulted into increased lag phase from 0 to 66 hours by *Pseudomonas fluorescens* [19]. Phenol is very toxic to most types of microorganisms at sufficiently high concentration and is not readily degradable. It can inhibit the growth rate even of those species that have the metabolic ability of utilizing it as a substrate for growth [20]. Goudar *et al.* [21] suggested that phenol concentrations greater than 1.3 g/L were toxic to the microbial culture. However, phenol exhibited inhibition to both specific growth rate and substrate degradation rate above 300 mg/L of initial phenol concentration [22].

One important concern is the toxicity of the organic chemical itself. Very often chemicals may be impregnable to biodegradation at low concentrations, yet may be toxic to the degrading population at higher concentrations, thus inhibiting their own biodegradation. Hence, the phenolic compounds are known to appear toxicity to bacteria [23].

*R. pyridinivorans* GM3 has ability to acclimatize the load of phenol, which demonstrates that presence of phenol in media upto 2.0 g/L does not adversely affect the viability. Acclimatization proved to overcome inhibition effect that usually occurred at high concentration of phenol because the biodegradability is limited due to toxicity is a function of concentration. If the concentration of the toxicant can be organized or the biomass is large enough, many highly toxic wastes or constituents can be biodegraded and by incrementally excess contaminant concentrations, more waste can be degraded by microbes. The key is to slowly increase the contaminant concentrations, allowing the microorganism population to adapt to the altering circumstances and produce the demanded enzymes and metabolites [24]. Phenol toxicity is always related with loss of cytoplasmic membrane integrity causing disruption of energy transduction, disturbance of membrane barrier work, inhibition of membrane protein function, and

subsequent cell death. After the addition of bacteriostatic concentrations of phenol, a dose-dependent efflux of metabolites such as ATP and K<sup>+</sup> ions was generated, as long as that glucose was provided as an energy substrate, a reaccumulation of K<sup>+</sup> ions at low phenol concentrations was shown [25].

Survival of bacteria often results from an inheritable resistance, also several environmental factors must be considered for the survival ability of microbes. *R. pyridinivorans* GM3 have strong adaptability to the environmental conditions, which observed it has high survivability and tolerability up to 7.0 g/L phenol concentration (Figures 2 and 3). From this result, it indicates that the adaptation of bacteria to phenol is necessary for the survival and biodegradation of the substrate in a medium with higher concentration of phenol. Microbes have mechanisms that enable them to tolerate toxic compounds at lethal concentrations. The strain of *Rhodococcus ruber* AC 239 was a good biosurfactant producer and was able to develop on several hydrocarbon sources as the biosurfactants have ability to decrease surface tension, blocking the creation of hydrogen bridges and certain hydrophilic and hydrophobic interactions [26].

Microorganisms have mechanisms that enable them to tolerate lethal concentrations of toxic compounds. Basile and Erijman [27] hypothesized that the dominance of particular bacteria at the different concentrations of phenol could be accredited to their functional traits related to the affinity and/or the tolerance to phenol concentration. This characteristic has been exploited in a wide range of bioprocesses that range from bioremediation applications to fine production chemicals. Kapoor *et al.* [28] studied tolerance capacity of four microorganisms namely *Pseudomonas aeruginosa*, *Yersinia* sp., *Serratia* sp. and *Pseudomonas fluorescens*. For different sublethal concentrations, they observed that phenol concentrations above 0.2 g/L was toxic to *Pseudomonas fluorescens* while others tolerated phenol up to 0.4 g/L. Visser *et al.* [29] reported that thirty-three strains of phenol-utilizing bacteria were isolated of which 31 were recognized. Most of the strains belonged to the genera of *Achromobacter*, *Clostridium*, *Azotobacter*, *Brevibacterium*, *Micrococcus*, *Flavobacterium*, *Pseudomonas*, *Bacillus* and *Sarcina*. After adaptation, many of these microorganisms could tolerate concentrations of phenol as high as 2400 mg/L. Whereas Rigo and Alegre [30] found that among 22 species of microorganisms isolated from wastewaters containing phenol, *Candida parapsilopsis* was found to be capable of growth on a medium with 1.0 g/L phenol. The phenol tolerance affects growth of bacteria; this points that they participate in the processes regulation, which are active within the growth and/or cell division. Single cell investigation data indicated that the cell division step of cell cycle is particularly susceptible to the toxic effect of phenol and its inhibition can be regarded as an adaptive response under conditions of phenol stress [13]. According to the literature, thirty soil bacterial isolates were screened and identified for phenol resistance. Four of these strains (belonging to genera *Bacillus*, *Staphylococcus*, *Proteus* and *Corynebacterium*) were resulted resistant to 15 mM (1.42 g/L) phenol [31].

The data suggests an effective bacterial inoculum size should be able to tolerate more concentration levels of phenol. It was evidenced that the tolerance of organism to phenol toxicity decreased with increasing concentrations. Also the results were emphasized that there is no difference between tolerability and survivability in these experiments, apparently the glucose dose not increase the phenol tolerance of *R. pyridinivorans* GM3 (Figures 2 and 3). On the contrary, Lob and Tar [32] reported 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 readily metabolizable carbon, as well as the cells can tolerate a much higher phenol concentration when grown in the presence of yeast extract.

Phenol is not readily degradable and is toxic to most types of microorganisms even at low concentrations [33]. The survival of a microorganism under the toxic effects of solvents depends on its ability to adapt to maintain the necessary biological functions. Sardesai and Bhosle [34] mentioned about the mechanisms of organic solvent tolerance involving novel adaptations such as the toluene efflux pumps, *cis-trans* isomerisation of membrane fatty acids, rapid membrane repair mechanisms. The *cis-to-trans* modification of the fatty acids studied here apparently is a new way of adapting the membrane fluidity to the presence of phenols, thereby compensating for the elevation of membrane permeability induced by these toxic substances [35]. Most of the organic pollutants may be susceptible to biodegradation at low concentrations; however, they may be toxic at higher concentrations, but *R. pyridinivorans* GM3 had strong adaptability, which showed high survivability and tolerability up to 7.0 g/L phenol concentration in the medium. The plasticity of the rhodococcal genome is a feature of these bacteria and genomic rearrangements are clearly evident. The role of insertion sequences cannot be in much doubt; however, other, as yet unknown, recombination mechanisms may play a vital role in the events observed, this will

ultimately be necessary for the continued biotechnological exploitation of rhodococci [36]. Hence, Zaitsev *et al.* [37] support the view that this genus may play an important role in the biodegradation of halogenated aromatic compounds in the environment. The genus *Rhodococcus* may be an important genus for use in the cleanup of the environment. Therefore, *Rhodococcus* spp. are increasingly becoming more important in the field of bioremediation and biotechnology due to their ability to degrade many pollutants and to produce biosurfactants or emulsifiers with beneficial applications [38].

### Conclusions

Phenol due to its toxicity, persistence and common occurrence in the biosphere, is one of the most important groups of eco-toxic compounds. The behavior and fate of phenol in the environment is of great concern for public health monitoring and environmental toxicology. The acclimatization ability of *R. pyridinivorans* GM3 to different concentrations load of phenol has been investigated. The *R. pyridinivorans* GM3 had strong adaptability to the environmental conditions, which showed that it has a high survivability and tolerability upto 7.0 g/L of phenol concentration in the medium. Obviously at 2.5 and 3.0 g/L of phenol concentrations with 1% inoculum showed inhibitory effect on the growth of *R. pyridinivorans* GM3 as pointed. However, the inhibitory effect of phenol on viability indicated that GM3 could not degrade 2.5 g/L phenol concentration with 1% inoculum size. The results showed its capacity to use *R. pyridinivorans* GM3 for biodegradation of phenol.

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