Lag phase and biomass determination of Rhodococcus pyridinivorans GM3 for degradation of phenol

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Abstract. Among various techniques available for removal of phenol, biodegradation is an eco-friendly and cost effective method. Thus, it is required to understand the process of biodegradation of phenol, such as investigate on lag phase and biomass concentration.

Phenol degrading bacteria were isolated from soil samples of industrial sites in enriched mineral salts medium (MSM) with phenol as a sole source of energy and carbon. One isolate of potential phenol degradation from consortium for phenol degrading studies was identified as Rhodococcus pyridinivorans GM3. Lag phase and biomass determination of R. pyridinivorans GM3 was studied with different phenol concentrations under pH 8.5 at temperature 32°C and 200 rpm.

Microbial biomass was directly proportional to increasing phenol concentration between 1.0 to 2.0 g/L with a maximum dry biomass of 1.745 g/L was noted after complete degradation of 2.0 g/L phenol in 48 hours.

1. Introduction
Phenol represents a serious ecological problem due to its wide spread use, toxicity and occurrence throughout the environment; hence, it is necessary to develop efficient strategies for its waste management [1]. Whilst Rigo and Alegre [2] reached that among twenty two microorganisms species isolated from wastewaters that containing phenol, Candida parapsilopsis had to be growth capable on a medium with phenol concentration (1.0 g/L). The degradation ability of Streptococcus epidermidis OCS-B was checked up to 200 mg/L concentration within 84 hours and so can be utilized the phenol by bioremediation of contaminated sites [3].

Much attention is paid on microorganisms that can completely degrade phenol, and there are a variety of phenol degrading cultures. Xanthobacter flavus MTCC 9130 can tolerate upto 1100 mg/L phenol concentration, the phase of lag elevated with the raise in concentration of phenol and the temperature of optimum growth was 37°C [4]. Shourian et al. [5] observed that Pseudomonas sp. SA01 degrades phenol at 0.7 g/L after an initial very short lag phase, then rapidly completed within 30 hours. Pseudomonas sp. SA01 was capable to degrade up to 1.0 g/L of phenol concentration and over concentrations of phenol (1.0 g/L) had a potential inhibitory affect growth of bacterial.
When an inoculum of bacteria is first introduced into medium, it will probably require a period to adapt to its new surrounding environment. Irrespective of variation of conditions and microbial strain, the increased phenol concentration leads to the increase in period of the lag phase, thus, extend the time of biodegradation. Moreover, the reduce in the degradation rate by Acinetobacter baumannii; on increase in the initial concentration of phenol from 125 mg/L to 1000 mg/L, an increasing lag phase period from 0 to 48 hours was observed and correspondingly extended the process of degradation from 84 hours to 354 hours [6]. The strain Corynebacterium sp. DJ1 granules had a lag phase of 12 hours at 2000 mg/L phenol concentration and degradation rate was 38.3, 36.4 and 34.7 mg/L per hour at concentration 1.0, 1.5 and 2.0 g/L respectively; however, phenol at 2500 mg/L inhibited microbial growth and degradation [7]. At higher concentration of phenol, the microbial growth inhibition was more and hence the lag phase time get longer for Alcaligenes faecalis to degrade 400, 700 and 1000 mg/L within 6, 12 and 26 hours respectively [8].

The elevated inhibition with the higher levels of phenol was obvious in longed lag period treatment of stream wastewater and can be negatively influenced by the co-pollutants existence [9]. Although the phenol is a poisonous composite which inhibits its possess change even at low concentrations, there are some methods accessible for phenol treatment; the biological treatment is mainly attractive because it has the significant to complete degrade phenol at often with generating minimum production of secondary waste and safe end products [10]. The simple recycle of the biomass by both, chemical and physical methods have been recognized for aromatic wastewaters treatment, but phenols biological degradation is progressively more being documented as a proficient and mild effective cost method [11]. Biomass is important parameter that affect phenol biodegradation rate, whereby it is correlated with differences of microbial growth stage, such as lag phase of microorganism during growth on media containing phenol Therefore, the objective of study is investigate lag phase and determination of biomass concentration of bacterial isolate during phenol biodegradation process.

2. Materials and Methods

2.1. Isolation
Samples were collected from 4 industrial and 4 agriculture sites in Hyderabad. Medium of enrichment for degrading bacteria was conducted to screen sample of soils under aerobic condition. Isolation of phenol degrading bacteria were carried out in enrichment culture containing 1.0 and 1.5 g/L of phenol by sub cultured into fresh MSM for several times. Among 26 isolates, one of the bacterial isolates that were evidenced high degradation of phenol and identified as Rhodococcus pyridinivorans GM3 by biochemical characteristics, morphological and microscopic 'Figure (1)'.

2.2. Growth medium
The mineral salts medium includes yeast extract (1.25 g/L), MgCl2.6H2O (0.35 g/L), K2HPO4 (0.35 g/L), Ca(NO3)2 (0.2 g/L ), FeCl2 (0.12 g/L ) and trace elements (0.2 mg/L of MnSO4. 2H2O, 0.2 mg/L of CuSO4.5H2O, and 0.1 mg/L of Na2MoO4, 0.1 mg/L of ZnSO4.7H2O) with phenol addition as source of sole carbon according to concentration assay[12].

2.3. Phenol estimation
Phenol was determined by method of direct photometric [13]. The supernatant was added 4-aminoantipyrene and using phosphate buffer (pH 6.8) and ammonium hydroxide (0.5N) for adjusted at pH 7.9 ± 0.1. Then, followed by oxidation with K2Fe(CN)6 and analyzed at 500 nm by spectrophotometer (Shimadzu - Japan) Visible/ Ultra Violet recording. The minimum detectable quantity of phenol in this method is 0.1 mg/L.

2.4. Phenol degradation
Phenol degradation was carried out on MSM (50 mL) containing 1.5 g/L of phenol concentrations (at triplicate) with 1% R. pyridinivorans GM3 of inoculation and incubated at 32°C, pH 8.5 and 200
rpm (optimization conditions) [14]. The samples were collected every 8 hours of interval from flasks containing phenol concentration 1.5 g/L respectively and phenol degradation was monitored.

2.5. Lag phase
For studying lag phase of *R. pyridinivorans* GM3 with phenol as sole carbon sources it was carried out in triplicates on MSM with different concentrations of phenol (0.5, 1.0, 1.5 and 2.0 g/L) at pH 8.5, 32°C and 200 rpm. Each phenol concentration was estimated at regular intervals of one hour of incubation.

2.6. Growth curve
MSM with 1% glucose was prepared in 250 mL flask and inoculated with 1% actively growing *R. pyridinivorans* GM3 and incubated at 32°C, pH 8.5 and 200 rpm. Growth was measured at regular intervals as culture optical density (OD) by spectrophotometer (Shimadzu) at 600 nm (in lab of Department of Microbiology- Science College- Osmania University).

2.7. Determination of biomass concentration
The biomass concentration was determined using the dry weight method. It was carried out on MSM (50 mL) including three phenol concentrations (1.0, 1.5 and 2.0 g/L) at triplicate in flasks (250 mL) and inoculated with 1% *R. pyridinivorans* GM3 and incubated at temperature 32°C, pH 8.5 and 200 rpm. For every six hours of incubation, biomass was estimated for all phenol concentrations. Fifty mL aliquots in plastic centrifuge tubes (100 mL) were centrifuged for 15 minutes at 5000 rpm [15] on 4°C. The samples were then rinsed (twice) with de-ionized water and at 105°C for 24 hours, the pellets were dried, cooled in a dessicator and reweighed. The dry weight of biomass as g/L was estimated by the difference between the first (empty) and the second weight.

![Figure 1. A: Light Microscopic picture of bacterial isolate GM3 with simple staining at magnification 1000X. B: Scanning electron micrograph of *R. pyridinivorans* strain GM3](image-url)

3. Results
The ability of *R. pyridinivorans* GM3 to degrade phenol in batch culture were studied by using MSM containing 1.5 g/L initial phenol concentrations with inoculum size 1%. It is clear from the results that *R. pyridinivorans* GM3 showed 100% degradation within 24 hours at phenol concentration 1.5 g/L which is shown in Figure 2. The results proved that *R. pyridinivorans* GM3 utilized/degraded phenol as sole carbon and energy source.
3.1. Lag phase of *R. pyridinivorans* GM3

The isolate bacterial *R. pyridinivorans* GM3 has ability to utilize phenol in MSM was used to study lag phase. Initial concentration of phenol acts a significant role in the process of biodegradation, since some contaminants of hydrocarbon, as well as phenol are recognized to possess inhibitory affect the duration of the lag phase of bacterium (Figure 3). The lag phases to initial 0.5, 1.0, 1.5 and 2.0 g/L phenol concentrations were 2, 3, 4 and 6 hours respectively. The results imply that the lag phase length elevated exponentially with concentration of phenol. The high phenol concentrations may have an inhibitory effect on growth of *R. pyridinivorans* GM3, hence reflected effect of toxic compound in the form of extended lag phase.

When a bacteria population is inoculated into a medium, the growth generally does not start immediately but after some time as known as the lag phase. In the lag phase there is a delay in the growth of bacterial population until the bacteria have become arranged to the surrounding conditions and source of nutrients.

![Figure 2](image_url)

**Figure 2.** Phenol degradation by *Rhodococcus pyridinivorans* GM3 with initial phenol concentration of 1.5 g/L.

**3.2. Growth curve**

Growth of GM3 was monitored by measuring turbidity at 600 nm (Figure 4). It is not clear that lag phase from the growth curve but there was less growth in first 4 hours. Growth curve of *R. pyridinivorans* GM3 showed a logarithmic phase extending up to 20 hours and maximum growth occurred at 20 hours (OD 1.324 $\approx 10^9$ CFU/mL), later the culture reached stationary growth.
3.3. Determination of biomass concentration
In order to determine biomass of *R. pyridinivorans* GM3 at varying phenol concentrations, freely suspended cells were inoculated (1%). Figure 5 shows biomass at 1.0, 1.5 and 2.0 g/L phenol concentrations, which were significant with phenol concentration and interdependence among the times needed for phenol degradation. At phenol concentration of 1.0 g/L the dry biomass found was lower than 1.5 and 2.0 g/L phenol concentrations. However, when the phenol concentration was 2.0 g/L it increased to approximately 1.745 g/L (dry biomass) and phenol could be completely degraded only at 48 hours. The biodegradation of phenol can be described by the non-elementary chemical reaction:

\[
\text{Phenol + Nutrients + } R. \text{pyridinivorans GM3 Cells } \rightarrow \text{More } R. \text{pyridinivorans GM3 cells + Products}
\]

**Figure 4.** Growth curve of *Rhodococcus pyridinivorans* GM3 in mineral salts medium

**Figure 5.** Biomass production of *Rhodococcus pyridinivorans* GM3 with 1.0, 1.5 and 2.0 g/L phenol concentrations

4. Discussion
The isolate *R. pyridinivorans* GM3 could degrade phenol with concentrations of 1.5 g/L in 24 hours. The results implied that GM3 has utilized phenol as sole source of carbon in MSM. Previous reports
suggest that *Rhodococcus* has the ability to degrade a variety of hydrocarbon and fuel additive compounds and could be efficiently used in bioremediation field for elimination of these compounds [16].

It was confirmed that lag phase depends upon the concentration of phenol in the media and when the concentration is increased, the lag phase period also extended concomitantly. The lag phase of *R. pyridinivorans* GM3 was obvious at 2.0 g/L phenol concentration within the first 6 hours of incubation, which might be due to the toxicity of phenol and the use of phenol as sole carbon and energy source. Similarly, Kim *et al.* [9] showed that increased inhibition of degradation with the higher levels of phenol was exhibited the lag phases increased. The correlations between concentration and lag phase imply that regarding inhibition of phenol degradation in kinetic analysis is useful for phenol degradation characterization [17]. Also, these results are in agreement with Oboirien *et al.* [18] whereby increasing initial concentration from 0.1 to 0.25 and 0.5 g/L enlarged the lag phase of *Pseudomonas aeruginosa* growth on pure culture to 8 and 16 hours respectively.

Phenol is not easy to degrade and is toxic to mainly microorganisms at adequately high concentration. The growth rate can inhibit even of those species that have utilizing it by metabolic ability as a substrate used for growth [19]. Since of the nature of phenol inhibitory to *R. pyridinivorans* GM3 populations at high phenol concentrations may completely inhibit bacterial degradation or caused in long lag times following a slightly rate of biodegradation. When increasing the initial concentration of phenol from 100 mg/L to 500 mg/L result to extend lag phase of *Pseudomonas fluorescence* growth from 0 to 66 hours [20].

Microbial bioremediation of a toxic chemical depends mainly on the ability of a microorganism to be viable, survive and tolerance to existing concentration and utilize that the compound as a substrate for growth. Microbial biomass was directly proportional to increasing phenol concentration between 1.0 to 2.0 g/L with a maximum dry biomass of 1.745 g/L was noted after complete degradation of 2.0 g/L phenol in 48 hours. If the toxicant concentration can be dominated or the biomass is big sufficient, numerous very toxic wastes or components can be degrade. Furthermore, by incrementally mounting concentrations of pollutant and waste in environment that can utilize the bacteria for removing this pollution. Growth of *R. pyridinivorans* GM3 varies depending on consumption of phenol and nutrient abundance. In other words, the activity of forming biomass indicated phenol degradation. Therefore, there is significant relation between phenol degradation and growth. Parameters such as concentrations of pollutant, microbial adaptation, tolerance and viable biomass are essential parameters that effect on biodegradation of phenol rate which further depend on the period of culture adapted to phenol [21]. Phenol is difficult to use as a substrate for growth within biodegradable process because the phenol inhibits the activity of innate of most kinds of microorganisms at lower and higher concentrations, also the phenol is toxic to microbial even at skimpy concentration [22]. Moreover, Goudar *et al.* [10] reported that the greater than 1.3 g/L of phenol concentrations were toxic to the culture of microbial. Conversely, phenol showed inhibition to degradation rate of substrate and specific rate of growth over initial concentration of phenol 300 mg/L [23].

In the present study, growth of *R. pyridinivorans* GM3 increased with increasing phenol concentrations. Degradation of organic substrates supplies bacteria with energy source and materials of building for metabolism, new cells growth, maintenance of cell. Attention was focused on phenol toxicity that inhibits growth depending on the concentration of phenolic compound to which microbial cells are exposed. Researchers have described results such as disruption of cell, precipitation of cell protein, inactivation of enzymes and leakage of amino acid from cells [24].

The tolerance of phenol effects on bacteria growth; this implied that they contribute in the processes regulations which are operating through the cell division and/or growth. The solution is to gradually increase the concentrations of contaminant, letting to the microorganism population to acclimatize to the altering conditions and generate the necessary metabolites and enzymes [25]. The step of cell division is mostly sensitive to effect of phenol toxic and its inhibition can be regarded as a response of adaption underneath phenol stress conditions [26]. Therefore, it is required to know the phenol biodegradation process that involved in degradation. Research has been conducted to understand biodegradation of phenol that are among the most prevalent and persistent environmental pollutants.
5. Conclusion

The degradation rates diminished or slowed considerably for length of lag phase when high concentrations of the phenol was used. Adaptation demonstrated that effect of inhibition was take place at high phenol concentration, hence the degradability is rimmed due to the toxicity is a concentration function. However, the rate of biodegradation depends on the state of biomass development. Obviously, the microbial biomass was directly proportional with increasing concentration of phenol.

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