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**RESEARCH ARTICLE**

# MAXIMUM PHENOL TOLERANCE AND SUBSEQUENT DEGRADATION PROFILE OF *ABACILLUS PUMILUS* STRAIN MCG 03AN ISOLATE FROM TANNERY WASTEWATER CONTAMINATED SOIL

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**ABSTRACT**

The present study emphasizes the phenol hyper tolerance and subsequent degradation behavior of a soil microbial isolate belongs to *Bacillus* genera. The bacterial species was isolated from tannery wastewater contaminated soil. Amongst the seven different bacterial isolate (MCG 01, 02, 03, 04, 05, 06 and 07), the isolate MCG 03 was selected and used to assess the phenol degradation efficacy at concentrations 500, 1000, 1500 and 2000 ppm. Isolate MCG 03 degrade 1000 ppm phenol within 24 h. Substrate inhibition was realized only at concentration >2000 ppm. The optimum experimental conditions determined were; pH 6.5; temperature 30°C, agitation 150 rpm. The growth kinetics studies revealed that the best fit with a Haldane model with specific growth rate, 0.01 $\mu_{max}$ $h^{-1}$andinhibition constant, KI 629.588 mg $L^{-1}$ with $R^2$ value of 0.991 compared to Edward model. It has been found that phenol degradation was effected through catechol dioxygenase enzyme system as evidenced through SDS- PAGE electrophoresis and zymogram techniques. Scanning Electron Micrograph displays increase in rod length of MCG 03 at higher concentration of phenol. In conclusion, the strain MCG 03 tolerates at 2000 ppm phenol concentration and suggested the suitability of this strain for bioremediation process.



## INTRODUCTION

Degradation of toxic chemicals through microbial methods is an approach accepted for bioremediation, since most of the degraded products found harmless. Phenol, a major organic pollutant in the effluents of petrochemicals, plastic, pharmaceuticals, coal refining, tanning and coke industries, demand immediate removal because of the toxic effects realized in humans and other living organisms (Kumar *et al*., 2013). The permissible level of phenol concentration according to the European Union recommendation for wastewater is 500 mg $L^{-1}$ and 500µg $L^{-1}$ for potable water (Christen *et al*., 2012). However, most of the industrial wastewater exhibited phenol concentration more than 5000 mg $L^{-1}$, which necessitates the suitable removal methods. The existing and the most effective biological methods of removal of phenol over chemical and physical methods also suffer because of the microbial growth inhibitory effect of phenol. Few recent research studies reported phenol removal up to the concentration of 500 to 3000 mg $L^{-1}$ from industrial wastewater or from simulated wastewater (Table 1) using diverse microbial species. Hypothetically, use of most of the effective species for remediation may in turn pose some pathogenic effect to other living organisms in long term use (not yet proved). Thus, a search for efficient and harmless phenol degraders is still continuing. Among the bacterial species Pseudomonads displayed effectiveness on par with yeast species, however, the tolerance level for *Pseudomonas* was <1000 mg $L^{-1}$. With regard to *Bacillus* sps, only few reports are available.

It has been understood that culture conditions play a major role in the degradation efficacy of the selected bacterial species. Further, in order to understand the effectiveness of the selected organisms, various inhibition models were reported with respective to bacterial species on specific growth rate and substrate inhibition, which in turn reveal the dynamics of microbial growth with reference to culture conditions (Banerjee and Ghoshal, 2010a). Furthermore, the analysis and the nature of the degraded products have also been considered in the assessment of effective degradation by the microbial species. The various instrumental techniques, which include, HPLC, LC-MS, etc., fulfill the requirements on the degradation studies. With these techniques, the degradation pathway chosen by the selected organisms can be identified, which will support the destruction of phenols or the toxic molecules. As described, in the present study, an attempt was made to have bacterial isolate from contaminated site which should have high tolerance and subsequent degradation of

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phenol at the maximum level of >1000 ppm. The effectiveness of the chosen isolate was determined based on growth profile, biomass, degradation products (HPLC), enzymes involved in the degradation (SDS-PAGE electrophoresis and zymogram techniques) etc. Other than nutrients, the role of environmental conditions on the maximum degradation of phenol concentration has also been assessed. Substrate inhibition models were made to understand the effectiveness.

## MATERIALS AND METHODS

### Microorganisms

### Isolation, identification and culture conditions

Isolation and identification of phenol degrading microbial species made using tannery effluent contaminated soil by enrichment technique followed by screening. The pure cultures obtained were stored at 4°C until use. Identification of the chosen isolate involves both phenotypic and genotypic characterization. Characterization studies starts with morphological, physiological and biochemical tests followed by 16srDNA gene sequencing. DNA was extracted according to the instructions given in Qiagen genomic DNA isolation kit and amplified by the universal primers 8F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-GGCTACCTTGTTACGACTT-3' as forward and reverse primers and amplified according to the method described by Turner *et al*. (1999). The PCR product was sequenced and the obtained gene sequences were compared to GenBank data bases. The growth medium used in the present study containing (g $L^{-1}$) Peptone – 5.0, Meat extract – 1.0, Yeast extract – 2.0, Sodium chloride – 5.0.

### Phenol degradation studies

To the pre-sterilized growth medium, filter sterilized phenol at concentrations 500, 1000, 1500 and 2000 ppm intended aseptically and incubated at 37°C with the pre-grown inoculum concentration of 1 x $10^7$ cells $mL^{-1}$ of the selected culture namely MCG 03. The residual phenol concentration, growth profile and the biomass concentration were determined at 2 h intervals for the period of 96 h for all the concentrations. Experiments without phenol have been considered for comparisons. Further, the biomass collected was subjected to extraction of intracellular enzymes according to the procedure summarized by Milo *et al*. (1999) and Nadaf and Ghosh (2011).

### Optimization of pH, temperature and agitation speed

Since, the degradation of organics depends on the culture and other environmental conditions, in the present study, role of pH, temperature and agitation speed were optimized for maximum degradation. Five different pHs (4, 5, 6, 7 and 8), three different temperatures (25, 30 and 35 °C) and five different agitation speed (100, 150, 200, 250 and 300 rpm) have been tested. Experiments were performed in 250 ml Erlenmeyer flasks with respective phenol concentration. All the experiments were performed in duplicate.

### Kinetics of cell growth and phenol degradation

Kinetics of cell growth with reference to phenol degradation was assessed using growth kinetics (Monad model) with two different inhibition models, namely; Haldane's kinetic model, and Edward's kinetic model(Wang *et al*., 1996; Monteiro *et al*., 2000; Kumar *et al*., 2005; Yan *et al*., 2005) general, cell growth and its kinetics in batch culture studies described as

$$\frac{dX}{dt} = \mu X - K_d X \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Since, the $K_d$ is negligible at an exponential phase, the growth can be represented as

$$\mu = \frac{1}{X}\ \frac{dX}{dt} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

By considering the inhibitory effect of phenol the following equation may be taken

$$\mu = \frac{\mu_{max} S}{K_S + S + \left(S^2 / K_i\right)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

In addition, the other inhibitory models like Edward's model also followed according to the following equation

$$\mu = \frac{\mu_{max} S}{S + K_S + \left(S^2 / K_i\right)\left(1 + S / K_S\right)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

From these models, $\mu_{max}$ $h^{-1}$, $K_s$ (mg $L^{-1}$) and $K_I$ (mg $L^{-1}$) were calculated and the best fit was obtained from the $R^2$ value.

### Analytical methods

Cell biomass was determined by measuring the cell dry weight (g $L^{-1}$). In brief, followed by growth, the experimental samples were subjected to centrifugation at 12000 rpm for 10 min at 4 °C. The cell pellet, thus obtained was washed with distilled water and dried at 105°C in hot air oven for the period of 48 h to have a constant weight and then weighed. The supernatant obtained was subjected to the determination of phenol and its degraded intermediates according to the procedure summarized below.

### Determination of Phenol

The total phenol concentration before the inoculation of MCG 03 and the residual phenol concentration after the growth of the organism at different time intervals were assayed both by spectrophotometric method (Yang and Humphrey, 1975; Oboirien *et al*., 2005) and HPLC analysis. In brief, for the spectrophotometric analysis, 10 µl of the cell free sample was mixed with 4-aminoantipyine, which reacts with phenolic compounds present in the sample and upon oxidation with potassium ferricyanide at pH 10.0 produces a purple-red colored end product and read at 500 nm.

### High performance liquid chromatography (HPLC) analysis

For HPLC analysis, the experimental conditions were as follows: HPLC analysis of the cell free sample made using C18 reverse phase column. The mobile phase was 0.01M

phosphate buffer (pH – 3.0 adjusted with phosphoric acid) with methanol and Tetrahydofuran (THF) (90:5:5 (v $v^{-1}$)) at 1 ml $min^{-1}$ flow rate. The chromatogram obtained was subjected to quantification from the standard curve made using phenol at the same operating conditions.

**Determination of intermediates (degraded products)**

The degraded products in the culture broth were determined spectrophotometrically and HPLC analytical methods. In brief, the representative degraded product, hydroxyl mucconic acid semialdehyde was quantified using analytical method summarized by Andrade *et al*. (2006). Similarly, for HPLC analysis, the cell free and filter sterilized samples made with the said instrumental conditions explained in the previous sections. Quantification made from the standard graph of Catechol run at the same conditions.

**Pathway elucidation**

**Enzyme analysis**

To assess the degradation pathway, the presence of enzymes responsible for degradation needs to be studied. In the present study, the presence of catechol di-oxygenases assessed using native PAGE-zymogram techniques. In brief, MCG 03 cells grown at different concentration of phenol was individually collected by centrifugation at 12000 rpm. The cell pellet obtained was washed with 0.1 M phosphate buffer and subjected to sonication (Lark Innovation Fine Technology, LTD., Chennai) for 4 min at 30s interval in the cool environment. The cell homogenate was centrifuged to remove the cell debris and the supernatant obtained was considered as crude enzyme. Total protein content (Bradford, 1976) and the molecular profile of the enzyme (Vilimkova *et al*., 2009) made using electrophoresis (10% separating and 3% stocking gel) under native conditions. The gel obtained was exposed to catechol (1%) for the period of 1 h to visualize the bands.

Parallel to electrophoretic assessment, the crude samples were subjected to C12D and C23 D activity assay according to the method summarized by Milo *et al*. (1999) and Nadaf and Ghosh (2011). In brief, the assay mixture contains 0.6 ml phosphate buffer, 0.3 ml distilled water, 0.1 ml catechol and 0.1 ml crude enzyme. The absorbance measurements were made at 260 and 375 nm, the characteristic absorbance of cis, cis muconate and 2-hydroxymuconic semialdehyde respectively. All the experiments were carried out in triplicates.

**Scanning Electron Micrograph (SEM) analysis**

Scanning electron micrograph for MCG 03 before and after degradation made according to the protocol summarized by Bozzola and Russell (1999) and using Hitachi S 4000 scanning electron microscope at the voltage of 10 keV.

## RESULTS AND DISCUSSION

Soil and sludge samples collected from the premises of common effluent treatment plant (CETP) located at Pallavaram and Ranipet, (Tamil Nadu, India) enriched with phenols for the period of 3 weeks and screening of potential species done in the presence of phenol concentration at 500 ppm. The rigorous screening methods offered seven potential species, namely MCG 01, MCG 02, MCG 03, MCG 04, MCG 05, MCG 06, MCG 07 and all the species shown growth in the presence of phenol. Fig. 1a depicts the morphology pattern of all the seven isolates. Further, the assessment on phenol degradation efficacy suggested that MCG 03 displayed appreciable phenol tolerance as well as degradation. During initial screening studies, MCG 03 shown growth at 1000 ppm under normal growth conditions and under optimized growth conditions MCG 03 displayed growth even at 2000 ppm as discussed in the following paragraphs. Identification of MCG 03 based on morphology, Gram staining, biochemical, 16s rDNA and SEM analysis revealed that the strain was pale creamy in colour, Gram +ve, rods, motile and not hydrolyzing the starch.

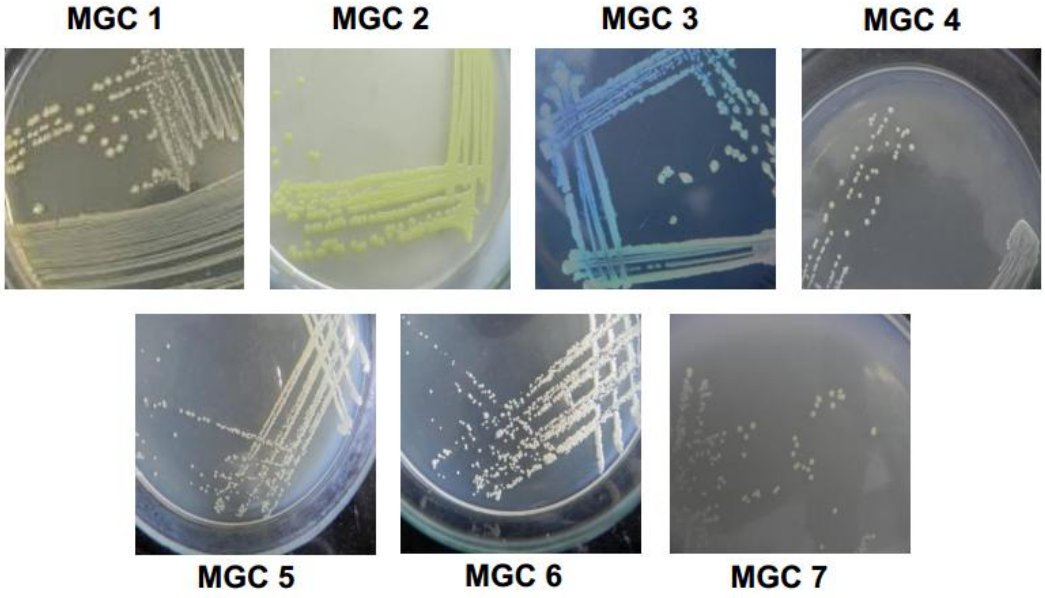


**Fig. 1a. Plate morphology of microbial strains screened for phenol degradation studies**

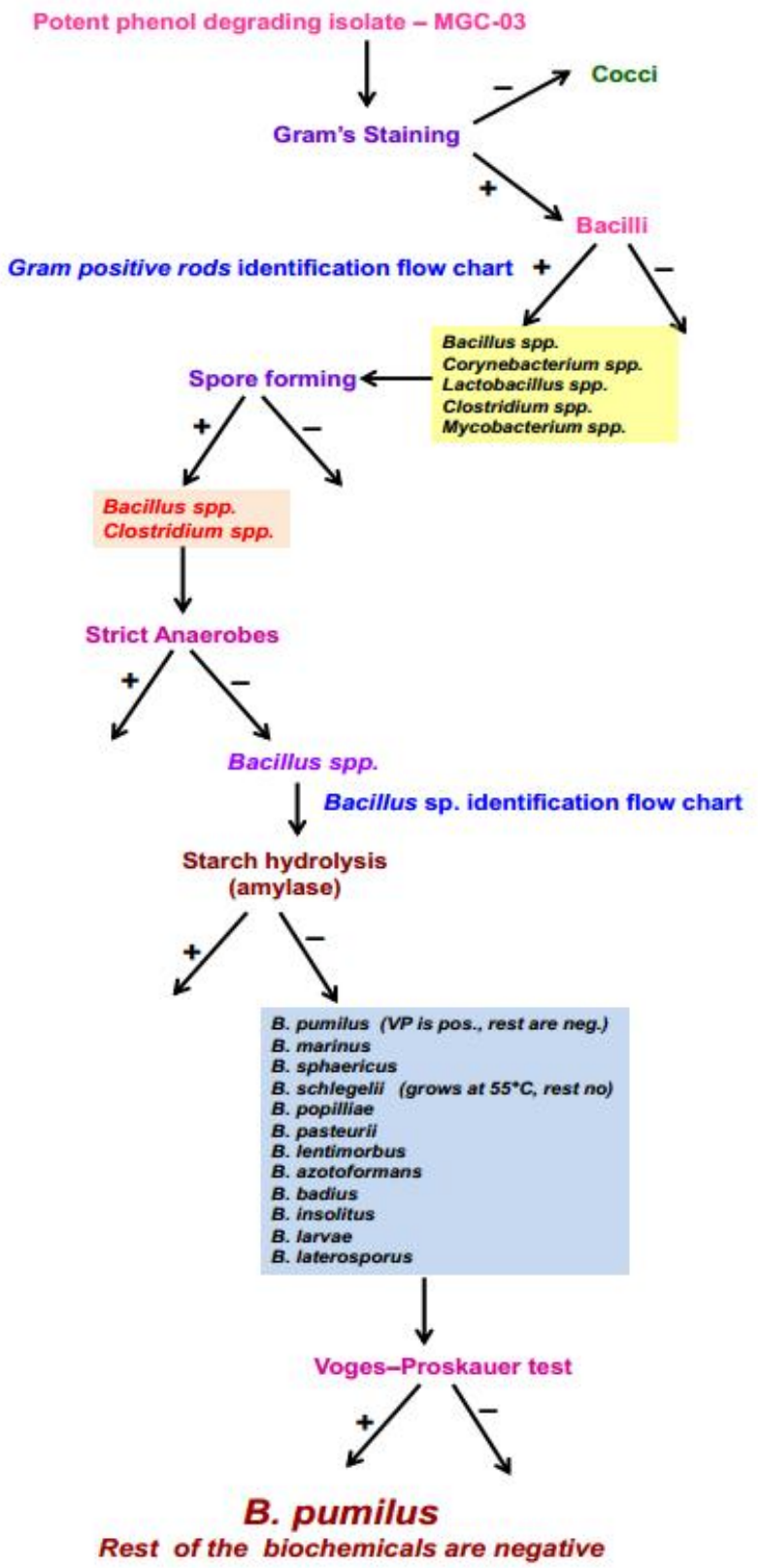


**Fig. 1b. Bergey's manual *Bacillus* identification chart – Biochemical analysis and flow chart of potent phenol degrading microbial isolate MCG 03**

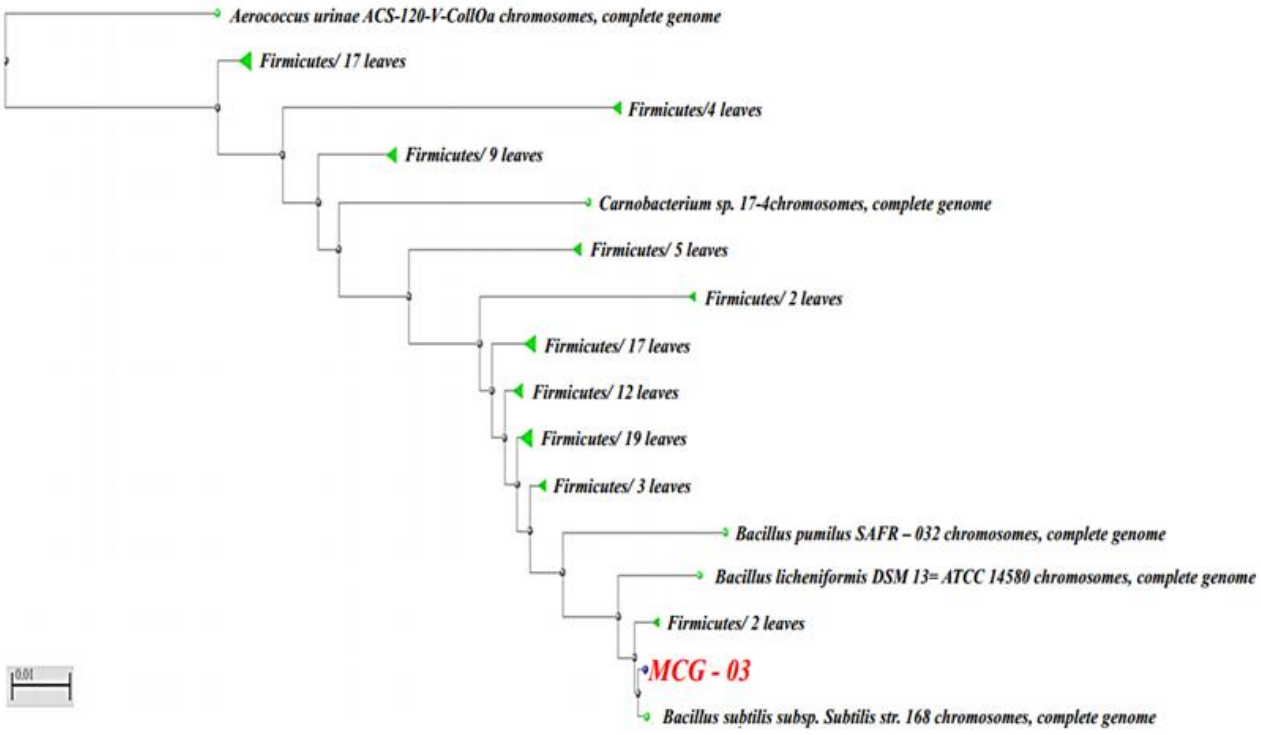


**Fig. 1c. 16S rDNA analysis of phenol degrading microbial isolate MCG 03**

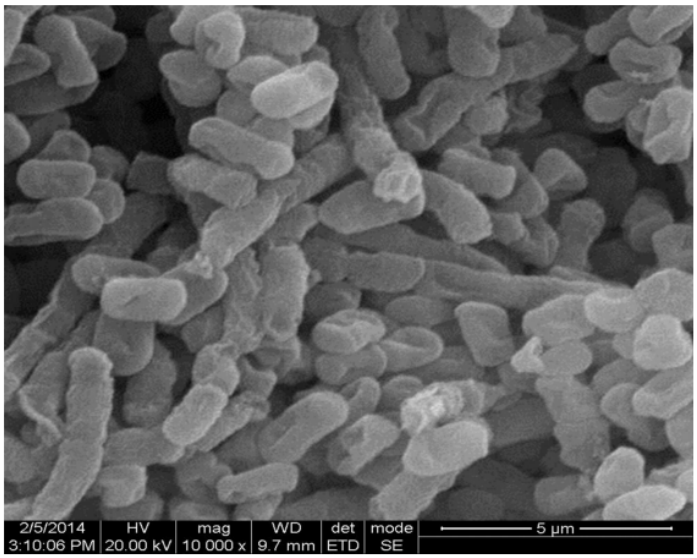


**Fig. 1d. Scanning Electron Micrograph (SEM) of potent phenol degrading microbial isolate MCG 03**

Table 2 summarizes the biochemical profile of MCG 03. Fig. 1b summarizes the Bergey's Manual *Bacillus* identification chart.16srDNA analysis of MCG 03 showed >94% resemblance with *Bacillus pumilus* (Fig. 1c) and thus the organism MCG 03 was named as *Bacillus pumilus* strain MCG 03. Fig.1d illustrates the SEM image of *Bacillus pumilus* strain MCG 03.

**Growth profile of strain MCG 03**

Fig. 2a depicts the growth profile of *B. pumilus* MCG 03 without and with phenol at different concentrations. The strain MCG 03 follow typical growth phase in the absence of phenol. However, in the presence of phenol at concentrations 100 and 500 ppm, the growth OD has shown an increase in the log phase compared to growth without phenol and suggested that MCG 03 utilize phenol as the source for the growth and metabolism. At 1000 ppm concentration, there was a negligible difference in the growth OD in comparison with without phenol. But, at 1500 ppm substantial decreases in growth OD was observed and at 2000 ppm concentration there was growth with no cell death. These observations suggested that at higher phenol concentrations, MCG 03 strain tolerate

well with the concentration up to 24 h. And, if the incubation period increases beyond 24 h, substantial increase in growth was observed, which revealed the hyper tolerance behavior of the strain MCG 03. Further, extended log phase till 22 h irrespective of the concentration of phenol could be reasoned to the slow acclimatization of organism to the intended phenol concentration. In addition, prolonged stationary phase till 48 h and extended death phase till 96 h were observed for all the concentrations studied.

When compared to other soil microbes, the strain MCG 03 displayed, tolerance towards phenol at concentration 2000 ppm and suggested the suitability of this organism in bioremediation of phenol in soils. As shown in Table 1 and according to the available literatures, the maximum tolerance at phenol concentration of 3000 ppm was exhibited by fungal species than bacterial species. The tolerance level of bacterial species was encountered as <1500 ppm, except *Corynebacterium* and *Flavobacterium*.

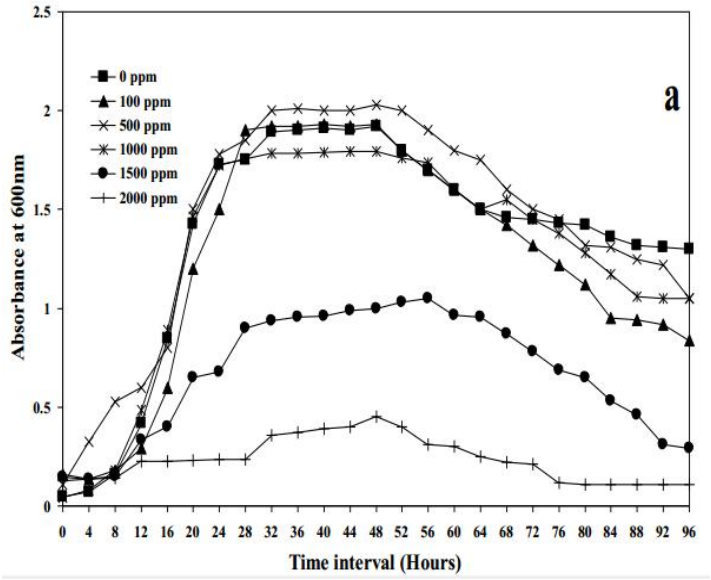


**Fig. 2a. Growth profile of *B. pumilus* strain MCG 03 with and without phenol at different concentrations**

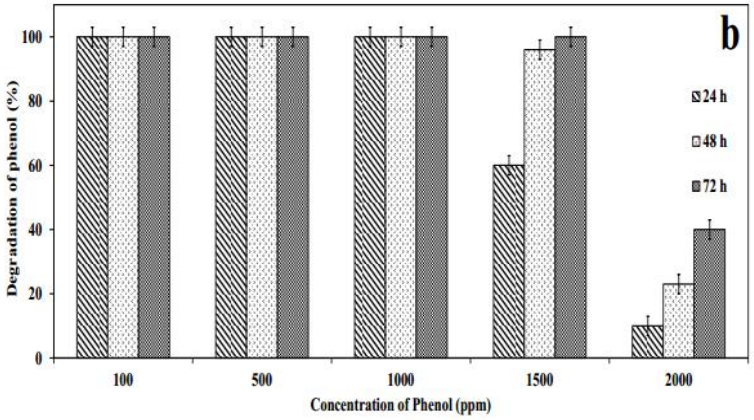


**Fig. 2b. Phenol degradation profile of the strain MCG 03 at different time intervals, 24, 48 and 72 h and at different phenol concentrations, 100, 500, 1000, 1500 and 2000 ppm**

**Table 1. Phenol tolerant profile of various microbial species explored in available literatures**

| Source | *Strain* | Phenol tolerance (ppm) | Reference |
|---|---|---|---|
| Fungi | *Fusariumsp* | 1000 | Santos and Linardi (2004) |
| | *Candida tropicalis* | 2000 | Yan *et al*. (2004) |
| | *Penicilliu mchrysogenum* | 1000 | Genge*t al*.(2006) |
| | *Candida tropicalis* | 3000 | Adave*t al*.(2007) |
| | *Candida tropicalis* | 1000 | Kuntiya *et al*. (2013) |
| Bacteria | *Pseudomonas putida* | 100 | Monteiro *et al*. (2000) |
| | *Bacillus spp* | 1400 | Ajaze*t al*. (2004) |
| | *Pseudomonas putida MTCC 1194* | 1000 | Kumar *et al*.(2005) |
| | *Pseudomonas sp* | 1250 | Pazarlioglu and Telefoncu (2005) |
| | *Acinetobacter* | 1500 | Adav *et al*. (2007) |
| | *Bacillus brevis* | 700 | Arutchelvan *et al*.(2006) |
| | *RWC-Cr1(Pseudomonas sp, Alcaligenes sp, Azotobacter sp)* | 1000 | Razali and Mailin (2006) |
| | *Corynebacterium* | 2000 | Ho *et al*. (2009) |
| | *Pseudomonas sp* | 1500 | Kaymaz *et al*.(2012) |
| | *Pseudomonas spp* | 250 | Shah (2014) |
| | *Flavobacterium* | 2000 | Zeng *et al*. (2014) |
| | *Pseudomonas fluorescens* | 1000 | Mahiudddin *et al*. (2012) |
| Yeast | *Aspergillus niger* | 300 | Supriya *et al*. (2014) |

**Table 2. The biochemical profile of MCG 03**

| Test | Results |
|---|---|
| Gram staining | Gram positive |
| Shape | rod |
| Motility | Motile |
| $O_2$ requirement | facultative anaerobes |
| CFU/ml | 6x10^7 |
| Voges Proskauer Test | + |
| Salt tolerance | 8.00% |
| Lactose | - |
| Maltose | + |
| Fructose | + |
| Dextrose | + |
| Galactose | - |
| Mannose | + |
| Maltose | + |
| Sucrose | - |
| Sorbitol | - |
| Inositol | - |
| Starch hydrolysis | - |
| Hemolysis | non hemolytic |

((+) Positive; (-) Negative)

However, in the present study, strain MCG 03 belongs to *Bacillus* genera demonstrated degradation of phenol upto 1500 ppm and tolerance upto 2000 ppm and suggested that the pre-exposure to the contaminants may be responsible for the hyper tolerance behavior of MCG 03. Fig.2b illustrates the phenol degradation profile of the strain MCG 03 at different time intervals (24, 48 and 72 h) and at different concentrations (100, 500, 1000, 1500 and 2000 ppm). Interestingly, at 100, 500 and 1000 ppm phenol concentrations, a complete removal of phenol from the growth medium was observed and suggested that 100% phenol removal within 24 h of incubation. Whereas, at 1500 ppm phenol concentration,60% removal was observed at 24 h and after 48 h the removal percentage rose to 96% and after 72 h it further rose to 100%. In the case of 2000 ppm concentration, the maximum removal of 40 % was observed after 72 h of incubation. Recently, Basak *et al*. (2014) reported degradation of 500 ppm phenol within 48h by *Streptrophomonas maltophilia*. Similarly, Kumari *et al*. (2013) found phenol tolerance and degradation capacity of *Rhodococcus pyrinidivorans* at concentrations of 500- 600 ppm. The survival of *Rhodooccus* sp at 1400 ppm phenol concentration was reported by Pannier *et al*. (2012). *Aspergillus fumigatus* from the Antarctic region shown complete degradation of phenol at 500 ppm concentration Gerginova *et al*. (2013).

With respect to experiments under different environmental conditions, in the present study, role of temperature on the degradation of phenol by the strain MCG 03 shown in Fig. 3a. With respect to phenol degradation > 99% removal observed at 25°C after 48 h of incubation compared to 30 and 35°C suggested that the optimum temperature for MCG 03 strain was 25°C. Similar to temperature, pH also plays a major role in the degradation studies. With the representative phenol concentration of 1000 ppm, phenol degradation studies carried out at pHs4, 5, 6, 7 and 8 suggested that maximum phenol removal was realized after 48 h at pH 6.0. The removal efficiency was very low at pH 4.0 and it slowly increases and reach the maximum at pH 6.0, then decreased at pH 7.0 and showed a moderate increase at pH 8.0 after 48 h of incubation (Fig.3b). In addition, pH of the growth medium during phenol degradation showed an increase to alkaline pH and suggested the buffering activity of the selected organism MCG 03, which may be considered for the tolerance behavior of MCG 03. With reference to agitation speed, degradation of phenol by the strain MCG 03, the maximum degradation was observed at 150 rpm for 1000 ppm phenol concentration after 72 h of incubation. Further increase in agitation reduces the percentage removal at a considerable level suggested that the optimum agitation speed for maximum phenol degradation by MCG 03 was at 150 rpm (Fig. 3c). Mordocco *et al*. (1999) reported that at pH 5.5 to 6.0, temperatures between 25° and 30°C, were found to be optimal for phenol degradation at low levels. Zhang *et al*. (2013) observed optimum temperature for maximum phenol degradation was in the range of 32- 42°C.

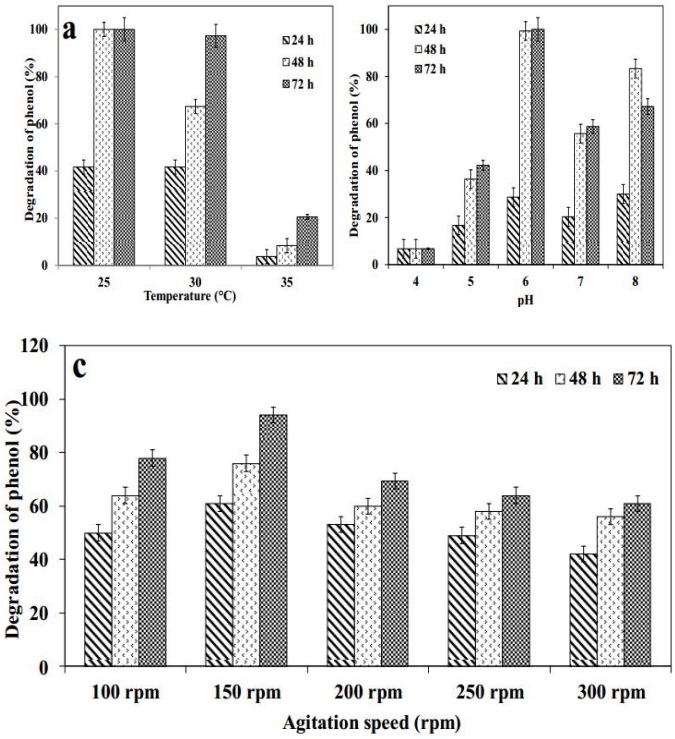


**Fig. 3. Optimization of pH (a), Temperature (b) and Agitation speed (c) for maximum degradation of phenol by MCG 03**

However, phenol degradation was maximum at 25- 37°C with *Acinetobacter sp*. RTE 1.4 according to Paisio *et al*. (2014). Chakraborty *et al*. (2010) found optimum temperature of 30°C for the native bacterial strains of coke oven processing wastewater. However, the observations made in the present study suggested that at the temperature of 25°C, the strain MCG 03 showed the maximum degradation. The maximum phenol degradation was realized at pH >6 in the presence of microorganisms like *Rhodococcus* (Zhang *et al*., 2013), *Arthobactercitreus* (Karigar *et al*., 2006), as reported found contradicting to the present study, where, we found the maximum degradation was at pH 6.0. Further, higher speed may damage the cells and reduces the cross-talk between the

cells and the medium components which impart metabolic stress to the organisms. Awan *et al*. (2013) found 120 rpm as an optimum agitation for the degradation of 90 % phenol. Fig. 4a illustrates the HPLC chromatrogram of standard phenol concentrations employed in the present study and Fig.4b (i, ii, iii, iv) depicts the chromatogram of the phenol concentrations 500, 1000, 1500 and 2000 ppm after 24 and 48 h of incubation. The reduction in peak intensity clearly indicates the reduction in phenol concentration and at the same time a small hump observed at 22.517 (not shown) could be reasoned to the formation of 2- HMSA according to Webb (1963).

With respect to kinetics, in the present study, to represent the degradation kinetics of phenol, several available kinetics models, such as Haldane's and Edward's models were found suitable for the experimental data obtained from the batch degradation experiments. The degradation rate, μ (1 $h^{-1}$) for those models are represented in Table 3.,where, S is the initial substrate concentration (mg $L^{-1}$), $\mu_{max}$ the maximum degradation rate ($1h^{-1}$), Ks thesubstrate-affinity constant (mg $L^{-1}$), and $K_I$ is the substrate-inhibition constant (mg $L^{-1}$). The degradation rate, μ was determined from the gradient of a semi-logarithmic plot of substrate concentration, S vs Time for each initial substrate concentration. $K_i$ is calculated using these models and represented in Table 3.

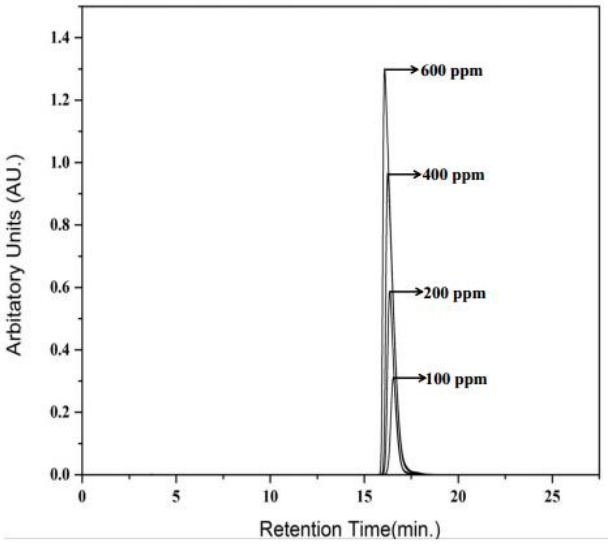


**Fig. 4a. HPLC chromatrogram of standard phenol at different concentrations (100, 200, 400 and 600ppm)**

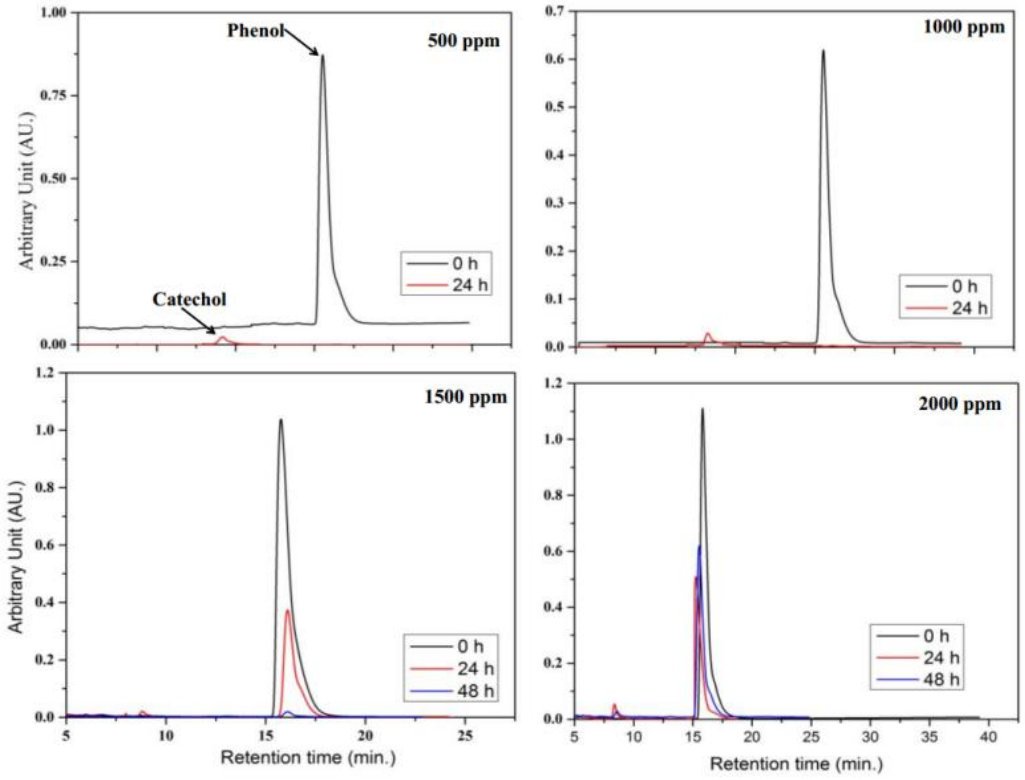


**Fig. 4b. HPLC chromatogram of the phenol concentrations 500, 1000, 1500 and 2000 ppm after 24, 48 and 72 h of incubation with MCG 03**

Larger the Ki value indicates the culture was less sensitive to substrate inhibition (Webb, 1963). Fig 5a, b and c displayed the specific growth rate of organism (Monad) and substrate inhibition models Haldane's and Edward's respectively. Both Haldane's and Edward's models predict phenol degradation trend satisfactorily. The regression coefficient value was 0.9 and 0.778, which predicts that Haldane's and Edward's respectively show a satisfactory degradation trend. The ($R^2$)

**Table 3. Specific growth rate and substrate inhibition parameters of the bacterial strain MCG 03**

| Strain | Model | $\mu_{max}h^{-1}$ | $K_s$ (mg $L^{-1}$) | $K_I$ (mg $L^{-1}$) | $R^2$ |
|---|---|---|---|---|---|
| *Bacillus* sp. | Monod Model $\mu = \frac{\mu_{max} S}{K_s + S}$ | 0.01 | 21.690 | - | 0.8355 |
| | Haldane Model $\mu = \frac{\mu_{max} S}{K_s + S + (S^2/K_I)}$ | 0.04 | 239.040 | 629.588 | 0.99124 |
| | Edward Model $\mu = \mu_{max} S \left[\exp\left(\frac{S}{K_I}\right) - \exp\left(\frac{S}{K_s}\right)\right]$ | 0.01 | $1.830\times10^5$ | 0.01 | 0.77827 |

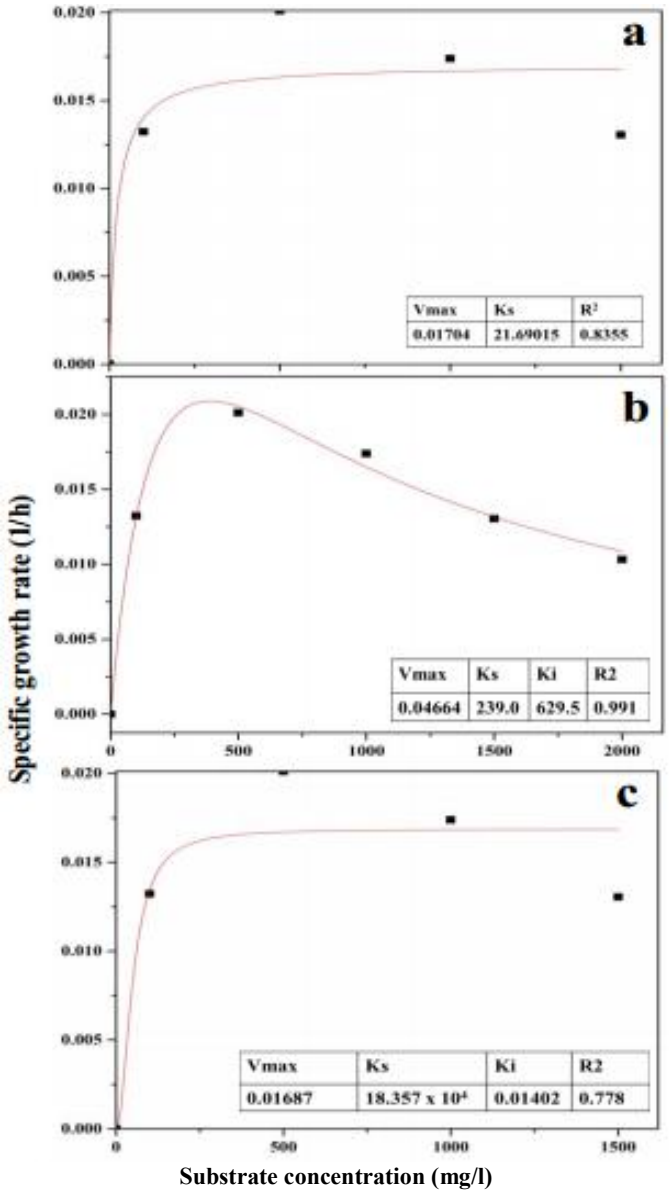


**Fig. 5. Growth kinetics parameter values of Monod's, substrate inhibition models: Haldane's and Edward's model on biodegradation of phenol using *B. pumilus* MCG 03**

correlation coefficient by Haldane's and Edward's model for phenol degradation using pure culture MCG 03 was 0.99124 and 0.778. For phenol degradation, the parameters for Haldane µm, *K*s and *K*i were 0.04 $1h^{-1}$, 239.040 mg $L^{-1}$ and 629.588 mg $L^{-1}$, respectively. The specific growth rate was achieved at phenol concentration of 400 ppm. The Ki value obtained as 629.588 shows the low inhibition of phenol on culture growth and could be reasoned to the contamination source Nuhoglu and Yalcin (2005). The value of Ks indicates that the organism can grow at low substrate level (Arutchelvan *et al*., 2006). Both Ks and Ki values suggested that strain MCG 03 able to grow in the wide concentration of phenol.

## Catalytic enzyme assays

In order to elucidate the phenol degradation pathway of the strain MCG 03, we determine the catechol dioxygenase activity in the culture broth. Fig. 6a depicts the SDS-PAGE pattern and native zymogram pattern of intracellular enzyme activity assay with reference to catechol 2,3 dioxygenase enzyme system. When the native protein interacted with the substrate the enzyme –substrate complex developed as a band as observed. The Lane L2, the SDS-PAGE profile showed two clear bands around 66 - 90 k Da and an additional band above 97 kDa. The Lane L3, the native gel showed a clear two active bands similar to SDS- PAGE pattern.

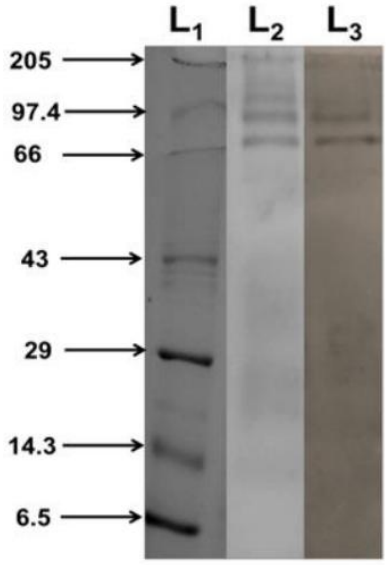


**Fig. 6a. Molecular profile of intracellular catechol 2,3 dioxygenase enzyme $L_1$- Molecular weight marker, $L_2$- SDS-PAGE Pattern, L3- Native Zymography PAGE pattern**

It has been well understood that metabolism of phenol by microorganisms was effected either thorough ortho or metapathway. The enzyme C12D catalyzes the orthopathway and the metapathway catalyzed by the enzyme C23D. Spectrophotometric analysis of C12D and C23 D at 270 and 375 nm is based on the formation of 2-hydroxymuconic semialdehyde (2- HMSA). In the present study, an increase in OD at 375 nm suggested that the degradation was effected through metapathway. An increase in phenol concentration, increase in OD at 375 nm suggested that effective release of catabolic enzymes even at higher concentrations.

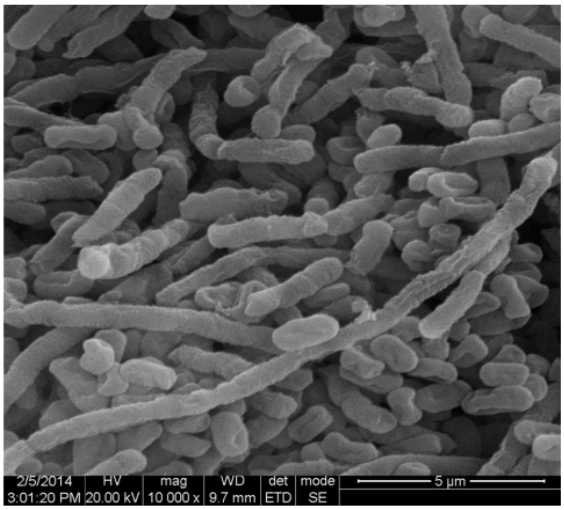


**Fig. 6b. SEM micrograph of growth of *B. pumilus* MCG 03 at higher concentration of 1500 ppm**

However, the absence of meta-pathway by *C.tropicalis* on phenol degradation reported by Banerjee and Ghoshal (2010b). Further, SEM analysis of the cultures (Fig. 6) after growth at higher concentration of 1500 ppm showed an increase in cell volume suggested the chemotaxis behavior of MCG 03(Bible *et al*., 2008).

## Conclusion

Degradation of phenol and related phenolic compounds using various microorganisms has been the topic of scientific interest for a number of decades. A large number of natural and synthetic organic compounds are biodegradable by microorganisms as part of their normal metabolism for energy and growth. Though more than 200 research articles discuss the phenol degradation by microorganism of varied genera, till this date, application of all the potential strains in soil remediation could not be achieved. The reason may be due to the instability of the organisms when they are exposed to the environment. Hence, a more stable phenol degrading microorganism is required to remediate the phenol contaminated soil or water. In the present study, an attempt was made using soil microbial species. The samples for isolation were collected from wastewater-contaminated soil. Enrichment technique followed in the present study offered a potential seven isolates with morphologically and biochemically distinct characteristics. Out of seven isolates screened, the study has been focused only to one isolate namely MCG 03, based on its growth at 2000 ppm phenol concentration.

The characterization studies revealed that the isolate belongs to *Bacillus g*enera with 94% similarity with *Bacillus pumilus*. The isolate was able to degrade 1500 ppm of phenol within 72 h of incubation and 40% phenol at 2000 ppm concentration in the same period of incubation. Optimization of environmental factors, viz., pH, and temperature and agitation suggested that MCG03 exhibit maximum degradation at pH 6.0, temperature at 25°C and agitation at 150 rpm. Above or below these said conditions, the degradation rate slows down significantly. The kinetics study revealed the best fit was with Haldane's model compared to Edward's model. With respect to the studies on identification of degradation pathway, the results showed, MCG 03 degrades phenol through meta-pathway and thus the metabolites are not toxic to cells. The chemotaxis behavior and the increase in cell volume suggested the tolerance behavior of MCG 03 towards phenol at higher concentrations.

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