Effect of physicochemical conditions on phenol degradation by Corynebacterium glutamicum: A focus on phenol catabolic enzymes

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The present study was aimed to investigate phenol catabolic activity and phenol tolerance ability of lysine producing bacterium, Corynebacterium glutamicum ATCC 13032. The results revealed that phenol inhibited the growth rate of bacteria (< 20%) at a concentration of >1500 ppm, indicating the toxicity of phenol to bacterial cells at higher concentrations. The maximum (> 95%) growth rate was observed at 200 ppm during a period of 24 h. In the present study, optimal conditions were standardized at different concentrations of phenol to know the phenol catabolism and phenol tolerance of C. glutamicum. Further, an attempt was also made to know the effect of yeast extract on phenol degradation by C. glutamicum. The results also demonstrated that increase in the yeast extract enhanced phenol (300 ppm) degradation efficiency of C. glutamicum, which reflects the phenol tolerance of bacteria. Thus, in-depth optimization studies on metabolic and molecular aspects may contribute to understand the potentiality of C. glutamicum in the biodegradation of phenol in large scale bio-remediation processes.

Keywords: Catechol 1,2-dioxygenase, C. glutamicum, growth, phenol, phenol hydroxylase

Introduction

In recent past, major concern has been shown towards the accumulation of man-made toxic chemicals including phenols in the environment. It is believed that improper treatment of industrial effluents and agricultural pesticides is one of the major sources of phenol pollution. The discharge of phenol and its related compounds target biogeochemical pathways of organic matter and nutrient cycling, and thereby interfere with the ecosystem equilibrium. Therefore, removal of phenols from the environment is of prime importance. Earlier, many approaches have been employed for environmental cleanup of phenol, such as, adsorption, chemical oxidation, solvent extraction and incineration. However, production of hazardous secondary products and economic constraints limited the use of these techniques. Currently, removal of organic pollutants using biological candidates, such as, microorganisms, is gaining importance and such an approach of implementing microorganisms is called bioremediation technology. Microorganisms are generally preferred because of their eco-friendly nature and cost-effectiveness. Although the exact mechanism of action of biodegradation is not thoroughly established, it has been suggested that during biodegradation, microorganisms degrade aromatic compounds through a range of intrinsic pathways and utilize them as energy sources, thereby detoxifying pollutants.

Several studies have suggested that microorganisms including Corynebacterium glutamicum have the potential to degrade aromatic compounds and utilize them as sole energy sources. C. glutamicum ATCC 13032 is well known for its lysine production and thus occupies a strategic position in pharmaceutical applications. In addition to its primary function, C. glutamicum has the ability to degrade various aromatic compounds including phenols. Recently, it has been suggested that C. glutamicum utilizes phenol as energy source and produces amino acids, such as, glutamate and proline, which have therapeutic values. Moreover, the knowledge of complete set of genome sequences of C. glutamicum ATCC 13032 provided the basis for identification of collection of pathways including aromatic acid mechanisms. Thus, it is apparent that C. glutamicum has the ability to grow in aromatic medium with enhanced aromatic catabolism. However, one of the major constraints in biodegradation is the toxicity of compounds on bacteria. Therefore, improvement of strains to tolerate...
more concentrations is advantageous at the perspective of ecotoxicology and environmental safety. It is well acknowledged that biodegradation principally depends on physico-chemical properties of aromatic compounds, because in order to assimilate aromatic compounds, the bacteria have to modulate their membrane and cell wall properties to the external environment. It is noteworthy to mention that among a range of factors, temperature, pH and time of incubation affect the bacterial cell adaptability to cope up with external environment. Hence, standardization of optimal conditions of bacteria is vital for complete mineralization and increasing the tolerability of bacteria towards aromatic compounds during biodegradation.

Considering the facts that, (a) the mechanism(s) of action of biodegradation of aromatic compounds including phenol are identified in C. glutamicum, and (b) few studies demonstrated the biodegradation of phenol by C. glutamicum, studies pertaining to the effects of physico-chemical factors on phenol catabolism by C. glutamicum are little exploited. Hence, the present study was aimed to investigate the degradation of phenol by C. glutamicum ATCC 13032 strain under different experimental conditions (time of incubation, temperature, pH and concentration of yeast extract) with an emphasis on metabolic events during phenol degradation.

**Materials and Methods**

**Chemicals**

Chemicals, such as, 4-dimethyl aminoantipyrene, and catechol were purchased from Kemphasol, Mumbai and Molychem, Mumbai, and NADH was purchased from Sigma Chemicals, USA. Analytical grade phenol was purchased from Sigma chemicals, USA. The desired concentrations of synthetic phenol solutions were prepared in sterilized distilled water before each experimental run. The solutions were always kept in brown flasks inside a dark cabinet to avoid photo-oxidation of phenol.

**Bacterial Strain and Cultivation Conditions**

*C. glutamicum* ATCC 13032 (America Type Culture Collection, Manassas, VA, USA), a wild strain, was selected as a test microorganism for the present study. The bacterium was cultivated on Luria-Bertani (LB) medium routinely at 30°C. The LB medium was changed to minimal salt medium (MSM) containing (1-1.2%) yeast extract as supplement and phenol (0 to 1500 ppm) as carbon and energy source. The cells were cultivated on a rotary incubator at 30°C and the growth of the bacterium was regularly monitored by determining optical density (OD) at 660 nm (OD) using a UV/VIS spectrophotometer (Thermo Scientific Evolution 201). To investigate the growth rate, the cells with a cell density of OD = 0.2 (per 10 mL phenol-MSM-1% yeast extract medium) were transferred to MSM from LB during experimental conditions. The agitation rate maintained was 150 rpm, throughout the experiment and also at different experimental conditions. All the results expressed represent the data from at least three independent experiments.

**Phenol Concentration**

To evaluate the tolerance of bacterium to phenol, different concentrations of phenol was used and inoculated with the bacterium. The initial concentrations of phenol tested were 0-1500 ppm. Based on the growth and degradation of the phenol, the optimum concentration of the substrate was selected for further experiments.

**Phenol Quantification**

During phenol degradation, phenol quantity was estimated spectrophotometrically using 4-amino antipyrene as colour indicator at an absorbance of 510 nm (OD). After addition of phenol (0-1500 ppm) to 10 mL suspension of cells at defined intervals, 1 mL sample was transferred to a reaction mixture containing 50 mL of 2N NH₄OH and 25 mL of 2% 4-amino antipyrene. The tubes were closed and the contents mixed. Again 25 mL of 8% K₃Fe(CN)₆ was added and the contents were mixed and centrifuged for 2 min. The absorbance of the supernatant was measured at 510 nm (OD). The phenol concentration was calculated by referring to the standard curve, prepared by using gradient concentrations of phenol (100, 200, 300, 400, 500, 600, 700, 800, 900 & 1000 ppm) and developing colour after adding regents and measuring absorbance at OD.

**Temperature, Time and pH Optima**

To determine the optimum conditions for the growth of *C. glutamicum* cells, experiments were...
carried at different physical factors, viz., temperature (30, 34, 37 & 44°C) and time of incubation (12, 24, 36, 48, 60 & 72h), and chemical factor, viz., pH (6.0, 7.0 & 8.0) in the MSM with phenol.

Enzyme Assays
Preparation of Cell-Free Extract
Cells freshly grown in MSM with phenol (200 ppm) were harvested by centrifugation (4000 rpm, 10 min) and the resulting pellet was washed twice with 0.33 M Tris-HCL buffer (pH 7.6). The cells were broken by using mortar and pestle at 15-20 strokes per min for 10 min and then cell debris were removed by centrifugation at 12000 rpm, 40°C for 20 min. The resultant cell-free extract was immediately used for enzyme assays, such as, phenol hydroxylase and catechol dioxygenases.

Phenol Hydroxylase Assay
Phenol hydroxylase activity was assayed spectrophotometrically (UV-Vis Thermo Scientific Evolution 201), following the disappearance of NADPH at 340 nm. In order to investigate endogenous oxidation of NADPH, the phenol substrate was omitted from the reaction mixture.

Catechol Dioxygenases
The cell-free extract was kept on ice and assayed as soon as possible for catechol dioxygenase activity using the method of Feist and Hegeman.

Catechol 1,2-Dioxygenase Activity
The ortho-cleavage product of catechol 1,2-dioxygenase was measured by the formation of cis,cis-muconic acid. The following reagents were added to a quartz cuvette: 2 mL of 50 mM Tris-HCL buffer (pH 8.0), 0.7 mL of distilled water, 0.1 mL of 100 mM 2-mercaptoethanol and 0.1 mL of cell free extract. The contents of the cuvette were mixed by inversion and 0.1 mL of catechol (1 mM) was then added and the contents mixed again. The absorbance was read at 260 nm over a period of 5 min and cis,cis-muconic acid formation was indicated by the increase in absorbance.

Catechol 2,3-Dioxygenase Activity
Catechol 2,3-dioxygenase activity was measured by following the formation of 2-hydroxymuconic semialdehyde, the meta-cleavage product of catechol. The following reagents were added to plastic cuvette: 2 mL of 50 mM Tris-HCl buffer (pH 7.5), 0.6 mL of distilled water, and 0.2 mL of cell-free extract. The contents were mixed by inversion and 0.2 mL of catechol (100 mM) was added and mixed with the contents. 2-Hydroxymuconic semialdehyde production was followed by an increase in absorbance at 375 nm over a period of 5 min.

Protein Concentration
The protein concentration in cell-free extracts was determined by the method of Bradford with bovine serum albumin as the standard.

Statistics
The data presented in the tables were the average of the triplicate readings obtained during experimentation. Data in Table 4 was statistically analyzed by performing one-way ANOVA using SPSS version 16. The data was considered significant at p< 0.05.

Results
Effect of Phenol Concentration on Bacterial Cell Biomass
The effect of phenol concentrations on growth kinetics of the bacterium was shown in Fig. 1. With increase in the concentration of phenol from 50 ppm to 200 ppm, bacterial growth increased significantly. Thereafter, a slight decrease in the growth rate was observed for the concentration 200-1400 ppm, showing a saturated growth response of bacterial cells. However, as the concentration of the phenol increased in the medium 1400-2100ppm, the growth rate was reduced below the original cell density. Thus, it is clear from the results that maximum growth of bacterial cells was observed at 200 ppm. However, for further studies, concentrations 100-400 ppm were selected.

Phenol Degradation Efficiency of C. glutamicum at Selected Concentrations
Phenol degradation assay by C. glutamicum was performed after 24 h of incubation and results are shown in Fig. 2. The bacterium showed the maximum
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Fig. 2—Effect of substrate concentration on phenol degradation ability of C. glutamicum.

Table 1—Effect of temperature on phenol degradation by C. glutamicum

<table>
<thead>
<tr>
<th>Phenol conc. (ppm)</th>
<th>Temp (°C)</th>
<th>Phenol degradation (%)</th>
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<tbody>
<tr>
<td>100</td>
<td>30</td>
<td>86.67</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>93.34</td>
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<td>37</td>
<td>86.67</td>
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<td>200</td>
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<td>40</td>
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<td>34</td>
<td>62.50</td>
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<tr>
<td></td>
<td>37</td>
<td>67.50</td>
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<tr>
<td></td>
<td>40</td>
<td>20.00</td>
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(96.67%) degradation efficiency at 200 ppm concentration of phenol. The phenol degradation ability of the bacterium at other concentrations was in the magnitude of 73.34 (100 ppm), 62.23 (300 ppm) and 52.47% (400 ppm).

Effect of Temperature, Duration and pH on Phenol Degradation Efficiency of C. glutamicum

The optimum conditions like temperature, pH and duration of incubation for the efficient degradation of phenol are shown in Tables 1-3. The effect of temperature on phenol catabolism by C. glutamicum is shown in Table 1. From the results, it is evident that the phenol degradation was active over a temperature range of 30 to 37°C. The bacteria showed the maximum degradation rate (95%) at 200 ppm phenol from 30 to 37°C. However, at 40°C, the efficiency was reduced to 56% for the same concentration of phenol, indicating the critical role of temperature. The percentages of phenol degradation obtained under different pH levels by C. glutamicum are shown in Table 2. From the results, it is clear that degradation
efficiency of *C. glutamicum* was the maximum at pH 7.0 for all the concentrations studied, but the catabolic rate decreased with the increase in the concentration of phenol and was, in the decreasing order, in the magnitude of 98.8% (200 ppm; 24 h) < 93.33% (100 ppm; 36 h) < 92.33% (300 ppm; 72 h) < 61.25% (400 ppm; 72 h).

Duration of incubation plays an important role in biodegradation processes. In the present study, degradation of phenol by *C. glutamicum* at different time points in a 3 day cut-off interval is shown in Table 3. Bacterial cells required 48 h (100 ppm), 24 h (200 ppm), 72 h (300 ppm) and 72 h (400 ppm) to obtain a degradation efficiency of 98.7, 98.8, 90 and 67.5%, respectively. From the results, it is clear that *C. glutamicum* required a shorter time period of 24 h to degrade 98.8% of 200 ppm phenol from the medium, whereas it required more than 3 days to degrade 67.5% of 400 ppm phenol. Thus, higher concentrations of phenol had adverse effect on the degradation efficiency of *C. glutamicum*.

**Effect of Phenol Concentration on Phenol Catabolic Enzymes**

**Phenol Hydroxylase**

Phenol hydroxylase activity was assayed in crude cell-free extract of bacteria grown in phenol-MSM with 1% yeast. Detectable phenol activity levels were observed at all phenol concentrations. However, the maximum enzyme activity was observed in cell-free extracts of bacterium grown in 200 ppm phenol-MSM medium containing 1% yeast extract (Table 4).

**Catechol Dioxygenase**

Table 4 also illustrates the activity of catechol dioxygenase in the cell-free extracts of bacteria grown

<table>
<thead>
<tr>
<th>Phenol conc. (ppm)</th>
<th>Phenol catabolic enzymes</th>
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<tbody>
<tr>
<td></td>
<td>Phenol hydroxylase (µmol/mg/min)</td>
</tr>
<tr>
<td>100</td>
<td>0.09 ± 0.002</td>
</tr>
<tr>
<td>200</td>
<td>0.15 ± 0.003</td>
</tr>
<tr>
<td>300</td>
<td>0.06 ± 0.004</td>
</tr>
<tr>
<td>400</td>
<td>0.03 ± 0.005</td>
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</table>

Values are mean±S.D. Mean values with different letters in a column differ significantly from each other at p<0.05.

on MSM at different concentrations of phenol. To distinguish between *meta* and *ortho* pathways, activities of characteristic enzymes, C23O (2,3 catechol dioxygenase) for the *meta* pathway and C12O (1,2 catechol dioxygenase) for the *ortho* pathway, were measured. The activity of C12O in crude extract of *C. glutamicum* indicated that the catechol ring fission was performed through the *ortho* pathways and not through the *meta* pathways.

**Effect of Yeast Extract on Phenol Catabolism**

The effect of yeast extract on phenol degradation was studied in order to know the effect of concentration of yeast extract (1% to 1.1%) on phenol catabolism by *C. glutamicum* (Table 5). The results of the present study show that supplementation of yeast extract (1.1%) to the phenol-MSM significantly increased phenol degradation at 300 ppm within 36 h. However, no changes were observed with other concentrations with the addition of 1.1% or 1.2% yeast extract.

The phenol degradation ability with respect to time at 300 ppm phenol reveals that addition of 1.1% yeast extract enhanced the phenol catabolism (90%) within 36 h time period (Fig. 3). The activity levels of phenol hydroxylase and catechol 1,2 dioxygenase levels were also significantly increased as compared to the

<table>
<thead>
<tr>
<th>Phenol conc. (ppm)</th>
<th>Yeast extract (%)</th>
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<tbody>
<tr>
<td>100</td>
<td>63.4 64.5 63.1</td>
</tr>
<tr>
<td>200</td>
<td>98.8 94.3 91.2</td>
</tr>
<tr>
<td>300</td>
<td>62.0 77.5 64.3</td>
</tr>
<tr>
<td>400</td>
<td>52.5 52.1 50.9</td>
</tr>
</tbody>
</table>

Fig. 3—Effect of yeast extract (YE) on phenol degradation by *C. glutamicum* cells grown in phenol (300 ppm)-MSM-yeast extract medium.
activity levels of both enzymes in cell extracts of bacteria grown in 300 ppm phenol-MSM containing 1% yeast extract (Fig. 4).

Discussion

Microbial degradation of toxic substances is one of the important growth areas of applied microbiology. It is well known that successful removal of any pollutant through microorganisms requires a detailed understanding of physico-chemical factors affecting biodegradation. Thus, the rationale of the present study was two-fold: 1) to obtain optimum physico-chemical conditions for effective degradation of phenol, and 2) to know the tolerability of bacteria towards phenol at selected concentrations.

In the present study, efficiency of phenol degradation was found to be the maximum between 30° to 37°C; however, 30°C was the optimum temperature for phenol degradation by *C. glutamicum* (Table 1). These results are in agreement with the earlier reports. The optimum pH for phenol biodegradation by *C. glutamicum* was 7.0. The pH was considered important factor because different bacteria degrade phenol at different pH values. For instance, *Halomonas campisalis* degrades phenol at alkaline pH between 8 and 11, whereas *Ewingella americana* degrades phenol at merely neutral pH 7.5. Further, *Klebsilla oxytoca* degrades phenol at acidic pH 6.8. Thus, it is evident that phenol catabolic enzymes respond differently to pH, which is believed to be species specific (Table 2). The response of protein profile of *C. glutamicum* ATCC 13032 to pH changes indicates an integration of the cell response to different stress conditions including xenobiotics.

The biomass of *C. glutamicum* was maximum at a concentration of 200 ppm in the MSM medium, while a decline was observed thereafter (Fig. 1). On the other hand, at concentrations greater than 1400 ppm, a reduction in the growth rate of bacteria was observed, which shows that phenol might be toxic at higher concentrations. The results are in concurrence with earlier reports. As the growth rate of >25% was observed at concentrations 100 to 400 ppm over a period of 24 h, further experiments were performed at these concentrations to know the maximum tolerable capacity of *C. glutamicum* cells at different experimental conditions.

As evident by degradation studies (Table 3), *C. glutamicum* tolerated and grew even up to concentration of 400 ppm phenol. It is believed that phenol cleavage pathway involves at least two critical steps. First, the aromatic ring being monohydroxylated by a multicomponent phenol hydroxylase at the ortho position to the pre-existing hydroxyl group and second, this is followed by aromatic ring cleavage either by catechol 1,2-dioxygenase (C12O), the ortho-pathway or catechol 2,3-dioxygenase (C23O), the meta-pathway. In the current study, an increase in the phenol hydroxylase activity was observed in crude extracts of bacteria grown in phenol-MSM as compared to bacteria grown on MSM (Table 4). This clearly suggests that presence of phenol in the medium triggers the phenol hydroxylase genes, thereby enhancing the activity of the enzyme. The results are in agreement with earlier reports, wherein it has been suggested that phenol degradation by *C. glutamicum* occurs through catechol branch of β-ketoacid pathway. Studies of Nardi et al. suggested that transcription level of phenol hydroxylase is induced considerably in the presence of phenol, whereas catechol 1,2-dioxygenase, which is a constitutive gene, is not greatly affected by the addition of phenol. The detectable levels of activities of phenol hydroxylase and catechol 1,2 dioxygenase in the supernatants indirectly supports the idea that there is a possibility of extracellular or membrane level phenol catabolism not permitting the phenol or initial metabolites into the cell.

The supplementation of 1.1% yeast extract to the medium containing 300 ppm phenol was catabolised by the bacteria with an efficiency rate of 90% under optimal conditions, such as, temperature (30-37°C) and pH (7.0), over a period of 36 h (Table 5). The results are in agreement with the earlier reports. Addition of yeast extract to the medium adjusts and balances the ratio between carbon and nitrogen, thereby maintaining proper ratio of C:N in the
medium. It has also been suggested that addition of yeast extract regulates the phenol catabolic enzymes to mitigate phenol toxicity.

Conclusion

From the present findings, it can be concluded that *C. glutamicum* degrades phenol at 200 ppm with an efficiency of >95% and also with maximum activation of phenol catabolic genes at experimental conditions, such as, a) broad range of temperature (30-37°C), b) medium pH of 7.0, and c) time of incubation of 24 h. Further, addition of 1.1% yeast extract enhances the phenol degradation ability of the bacteria.

Acknowledgement

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References