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# ISOLATION, PURIFICATION AND CHARACTERIZATION OF CATECHOL 1,2-DIOXYGENASE FROM *PSEUDOMONAS* SP.

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**ABSTRACT:** Phenol degradation bacteria which was isolated from the effluent canals of Vizag steel plant was characterized in the present study. Biochemical and morphological studies reveals that the strain belongs to *Pseudomonas* SP and it was named as *Pseudomonas* sp strain DS 002. The strain used phenol as source of carbon source and optimum growth was noted at 3 mM concentration of phenol at 8hrs. Biodegradation pathway for phenol in *Pseudomonas* sp. strain DS 002 was elucidated based on growth studies, enzyme activities, oxygen uptake studies and TLC. It also capable of make use of *p*-nitrophenol and *cis*,*cis*-muconic acid as carbon source. *Pseudomonas* sp. strain DS 002 degraded the phenol via catechol and *cis,cis*-muconic acid following *ortho*-cleavage pathway. Native catechol 1,2-dioxygenase from *Pseudomonas* sp. strain DS 002 was purified and its molecular weight was 32 k Da. The effect of substrate concentration on native catechol 1,2-dioxygenase was studied and was found to be 100 µM concentration of catechol and has wide range of substrates which include 3-methyl catechol, 4-methyl catechol, 3- Isopropyl catechol and pyrogall. The optimum catalytic activity of catechol 1,2-dioxygenasewas observed at the temperature of 37ºC and $p^H$7. Metal ions like silver nitrate (88%), copper sulphate (72%) and ferrous sulphate (4%) inhibited the activity of catechol 1,2-dioxygenase. Sulfhydril agents like PCMB (20%), N-Ethyl malemide (5%) also inhibited the activity of catechol 1,2-dioxygenase whereas DTNB and *O*-phenanthroline does not show any effect. In addition chelating agents like EDTA (5%) also inhibited the activity of catechol 1,2-dioxygenase.

**KEYWORDS:**Phenol degradation bacteria, *cis,cis*-muconic acid, Catechol 1,2-dioxygenase.

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## 1.INTRODUCTION

Pollution can be defined as any undesirable changes in physical, chemical and biological constituents of air, water and soil to the extent of harming the living organisms, damaging the monuments of our cultural heritage, affecting natural resources and interfering with comfortable existence. Pollution is one of the major challenges of today's civilization [1]. For centuries human beings believed that atmospheric, terrestrial and aquatic systems are sufficient to absorb and break down various pollutants released from industry and farming which we now feel as not true. Today the production and use of organic compounds including chemical fertilizers and pesticides have increased enormously with the growth of industrial activities leading to the accumulation of synthetic organic chemicals as environmental pollutants. For obtaining high yields of good grains, the use of chemical fertilizers, pesticides has become indispensable and the use of pesticides has become an integral part of modern agricultural systems. It is extremely difficult to estimate the pesticide quantity released into the environment during pesticide production, formulation and application. The quantity applied to crops, only 1% pesticide is utilized while the remaining 99% goes directly into soil, indirectly into water, biota and food chain leading to pollution problem [2] [3]. In addition, the manufacture, transportation and distribution of petroleum and chemical products have resulted in hydrocarbon contamination, which is also a major environmental problem.Phenol is one of the most widely used organic compounds in existence and is a basic structural unit for a variety of synthetic organic compounds including agricultural chemicals and pesticides. Phenol is naturally found in decaying dead organic matters like rotting vegetables and in coal. The German chemist, Runge isolated phenol from coal tar in 1834 and named karbolsaure (coal-oil acid orcarbolic acid), though its composition was not known until 1841. Phenol (hydroxy benzene) is both a synthetically and naturally produced aromatic compound. It was first used in the raw state, as creosote, to prevent the weathering of railway ties and ship's timber, and to reduce the odor of decomposition in sewage. Phenol and its derivatives are widespread in chemical industry based on processing of resins into plastics, disinfectants, pesticides, paints, anti-oxidants and perfumes. Additionally, phenol is a common pollutant to agricultural activities due to its presence in the industrial effluents. Phenol is a toxic and hazardous substance even at low concentrations [4] [5] and efficient treatment methods are necessary to reduce phenol concentration in wastewater to acceptable levels. The toxicity and environmental recalcitrance of phenol and its derivatives have promoted studies on their degradation by microorganisms.Phenol and its derivatives are highly toxic and hazardous to the living organisms. Phenols may be fatal by ingestion or skin absorption, since it quickly penetrates the skin and may cause severe irritation to eyes and respiratory tracts. It is listed among priority organic pollutants by the US Environmental Protection Agency [6]. Phenol is a potential in human carcinogen and of considerable health concern, even at low concentration. Hence, there is a need for the treatment of waste water containing phenol. The removal of such hazardous

organic pollutants from waste water is a growing issue [1], as water is a precious commodity [7] [8]. In this regard, industrial effluents containing phenols require proper treatment prior to discharge into the environment [9]. Many technologies have been investigated for removing and degradation of phenolic compounds in waste water. These include adsorption, chemical oxidation and biodegradation [10]. But methods such as solvent extraction [11], activated carbon absorption and chemical oxidation often suffer from serious draw backs including high cost and formation of hazardous byproducts. Among various methods available, biodegradation is economical and environmental friendly. Biological treatment of phenol has therefore been an increasingly important process in pollution prevention. In the steel plants, high quality of coal is required to extract iron from the ore. In order to improve the quality of coal it has to be heated to remove phenolic compounds and water from the inferior variety of coal. During this process a lot of phenolic vapors escape into the air polluting the atmosphere. Strategies have been developed to trap the phenolic vapors through cold water. Though most of the phenolic compounds are trapped in this process, disposing and decontaminating the water is the major problem. Certain industries that follow good Safety, Health and Environmental (SHE) practices have treatment plans to remove the toxic phenolic wastes from the effluents. However in some industries no SHE practices are followed and they pollute almost all components of the environment causing unexplainable miseries to the inhabitants surrounding the industrial units. To keep this in view in our lab we isolated and characterized a novel strain which degrades the phenol compound to develop bioremediation strategies.

## 2. MATERIALS AND METHODS

### Isolation of phenol degrading bacterium through enrichment culture technique

Bacteria were isolated by enrichment culture technique using phenol as sole source of carbon. Soil samples from the banks of effluent canal of Vizag Steel plant, Visakhapatnam, Andhra Pradesh, India were collected, air-dried in a cool dry environment and stored in similar condition until further use. For isolation of phenol degrading bacterial culture, 10 g soil sample was taken in 250 ml flask containing 50 ml of M9 medium and 3 mM of phenol as sole source of carbon. The contents were then incubated at 30ºC in an incubator shaker for seven days. Different colonies were selected several transfers were made on M9 medium containing phenol as sole source of carbon.

### Morphological studies of *Pseudomonas* sp. strain DS 002 by Scanning Electron Microscope

The bacterial suspension was filtered (0.45 μm pore size) and fixed on the membrane with 10 ml of 3% glutaraldehyde fixative in 0.1 M phosphate buffer ($p^H$ 7.4). The fixative was left in contact with the cells overnight at 4˚C. The fixed cells were washed with buffer and post fixes for 1 h in 1% osmium tetra oxide in phosphate buffer. Filter was rinsed with buffer and dehydrated through a series of ethanol solutions with increasing concentrations (50, 70, 95 and 100% ethanol). Ethanol was replaced with liquid $CO_2$ and the sample was dried in a critical point dryer. Cells were sputter coated with gold-palladium and examined in a Phillips XL-30 Scanning Electron Microscope at 20

**Growth behavior of *Pseudomonas* sp. strain DS 002 using Phenol as sole source of carbon**

Growth studies were carried out independently by growing the bacterium in M9 medium having phenol (1 to 5 mM), *p*-nitrophenol (0.5 to 2 mM), *cis,cis*-muconic (0.5 mM), catechol (0.5 mM) and hydromuconic semialdehyde (HMSA) as sole source of carbon. The growth was monitored spectrophotometrically at $A_{600}$ by collecting the culture at two hour intervals for a period of 24 h. A growth curve was plotted by taking OD on Y-axis and time scale on the X-axis.

**Estimation of protein and Enzyme assay**

The protein present in the cell free extract was quantified by following standard protocol. Catechol-1,2 -dioxygenase assay reaction mixture contained 50 µl of 50 mM Tris-HCl buffer, (pH 7.5), 50 µl of cell free extract and 10 µl of 100 µM concentration of catechol as substrate and 890 µl of double distilled water and the absorbance was measured at 260 nm. The specific activity was measured as µ moles of *cis,cis*-muconic acid formed /min/ mg of protein [12]. Catechol 2,3-dioxygenase assay reaction mixture contained 50 µl of 50 mM Tris-HCl buffer, (pH 7.5), 50 µl of cell free extract, 10 µl of 100 µM concentration of catechol as a substrate and 890 µl of double distilled water. The contents were incubated at 37ºC for 30 min and absorbance was measured at every 30 seconds up to 5 min and absorbance was measured at 375 nm. The specific activity was mentioned as µ moles of HMSA formed/min/mg of protein.

**Oxygen uptake studies of *Pseudomonas* sp. strain DS 002**

An oxygen uptake study was carried out by using polarigraphically using an oxygraph. The bacterial cells grown to log phase, were collected and washed twice with 50 mM potassium phosphate buffer ($p^H$ 7.0) and the cells were carefully diluted with phosphate buffer so as to obtain an O.D. of 0.6. The oxygen consumption was measured by adding the stock solution of possible catabolic intermediates of phenol such as catechol, to the reaction cell at a final concentration of 0.05 mM (50µl) using a Hamilton syringe. The oxygen consumed by the cells was measured as nanomol/mg/min [13].

**Extraction and identification of intermediary metabolites of phenol degradation**

The strain DS 002 was grown to reach the O.D of 0.5 in M9 medium supplemented with phenol as sole carbon source. The spent medium was collected by removing the cells after centrifuging the culture at 6000 rpm for 10 minutes. Then the $p^H$ of the spent medium was adjusted to 2 with 6 N HCL and followed by extraction of metabolites with ethyl acetate and the residue was dissolved in minimal volume of methanol and stored in -20˚C until further use. TLC plates with 0.5 mm thickness were prepared by spreading silica gel slurry (40 gm silica gel/100 ml of double distilled water) over the clean glass plates. The methanol dissolved metabolites were analysed by using TLC plates. The solvent system used for separation of phenol degradation products was Acetone:Benzene:Water in a ration of 1:1:2. After separation the TLC plates were air dried and gently sprayed with Folin-

Ciocaltaeu reagent for detecting the metabolites.

**Kinetic properties of Catechol 1, 2-dioxygenase**

Effect of substrate concentration on Catechol 1,2-dioxygenase was studied by using the reaction mixture (940 µl of 50 mM Tris-HCl buffer ($p^H$ 7.5), 50 µl of purified enzyme) and 10 µl of catechol (5 µM to 200 µM) as substrate. The contents were incubated at 37˚C for 30 min and activity was observed every 30 seconds up to five min at 262 nm. The effect of substrate analogues on catechol 1,2-dioxygenase was studied using different substrate analogues such as 4-Methylcatechol (1.0 mM), 3-Methylcatechol (0.1 mM), 3-Isopropyl catechol (0.1 mM), and pyrogallal (0.1 mM).

**Effect of temperature and $p^H$ on Catechol 1,2-dioxygenase**

Catechol 1,2-dioxygenase activity was measured in the temperature range 10-80ºC to determine optimum temperature. The effect of $p^H$ on enzyme activity was studied at $p^H$ ranging from 3-10, using citrate phosphate buffer ($p^H$ 3.0-5.0), sodium phosphate buffer ($p^H$ 6.0), Tris-HCl buffer ($p^H$ 7.0-8.0), and glycine NaOH buffer ($p^H$ 9.0-10.0).

**Effect of metal ions, sulfhydryl agents and chelating agents on the enzyme activity**

The effect of metals like $AgNO_3$ (0.1 mM), $CuSO_4$ (0.1 mM), sulfhydryl agents like PCMB (*p*-chloromercuribenzoate) (0.5 mM), N-Ethyl Malemide (0.1 mM), DTNB (5,5-dithiobis-2-nitrobenzoic acid) (0.1 mM), and chelating agents like *o*-phenanthroline (1.0 mM), Tiron (0.1 mM), α-α, Bipyridyl (0.1 mM) was studied on the activity of catechol 1,2- dioxygenase.

## 3. RESULTS AND DISCUSSION

**Isolation of phenol degrading bacteria**

Phenolic compounds are common constituents of many industrial effluents. These compounds are toxic by ingestion, contact, or inhalation, even at low concentrations. In treating phenolic compounds, the biological method has attracted more attention than physical and chemical methods because many different types of microorganisms are known to utilize phenol as their sole source of carbon and energy. Biodegradation of phenolic compounds by bacteria has been extensively studied and a large number of phenol-degrading bacteria have been isolated [14] [15]. In this study we have isolated a phenol degrading bacterium through selective enrichment culture technique from the soils contaminated with effluents of Vizag Steel plant using phenol as sole source of carbon. Pure isolated colonies were observed in and as evidenced by the biochemical features, gram staining it was gram negative, rod shaped and the isolated bacterium was identified as *Pseudomonas* sp. was designated as *Pseudomonas* sp. strain DS 002. Biochemical characterization results revealed that the bacterium was positive for oxidase, catalase and gelatin hydrolysis and negative for urease test, hydrogen sulfphide test, indole, glucose fermentation, Methyl red test, citrate test and Voges-Proskaruer test. Since the time of isolation, the *Pseudomonas* sp. strain DS 002 was always maintained in M9 medium containing 3 mM phenol. *Pseudomonas* sp. strain DS002 was grown both in the solid media as well as in broth. In both the cases the strain has effectively used the phenol

as sole source of carbon. The scanning electron microscope is a valuable tool to light and transmission electron microscopy for studying gross cell morphology. The isolated bacterium structure was shown in Figure 1A. The bacterium has rod shaped structure and measuring size up to 2.45 µm at the magnification of 25600X. Gopaul *et al*., (1991) [16] isolated a phenol degrading psychotropic strain *Pseudomonas putida* Q5. The degradation kinetics was studied at 10˚C and the phenols were degraded by 65 to 80 percent at an initial concentration of 500 ppm. They concluded that psychotropic strains are economical for wastewater treatment process in cold climatic region. Likewise the strain *Penicillium* Bi712 isolated from the contaminated soil in Bitter field (East Germany) could utilize phenol as the sole source of carbon and energy and tolerated up to 1500 ppm [17]. Yeast are also degraded the phenol and they were isolated from the soil yeast strains are A01, B02 and L0. B02 degrade phenol at 500 to 700 ppm where as A01 and L02 degrade phenol at 1000 ppm [18].

**Effect of phenol on growth of *Pseudomonas* sp. strain DS 002**

In present study, the bacterial strain was grown at different concentrations of phenol ranging from 1 to 5 mM. *Pseudomonas* sp. strain DS 002 has effectively utilized phenol as the sole source of carbon and energy. In 1 mM of phenol the bacterium grew maximum to 0.16 OD at 4 h, in 2 mM phenol the bacterium grew maximum 0.24 OD at 6 h, in 3 mM of phenol the bacteria grew 0.45 OD at 8 h, in 4 mM of phenol the bacterium grew 0.38 OD at 12 h and 5 mM of phenol the bacterium grew 0.28 OD at 20 h. Among the concentrations of phenol tested, 3 mM was the optimum concentration for growth of *Pseudomonas* sp. strain DS 002. A typical growth curve was drawn and presented in Figure 1B. The growth curve suggests that there was an initial lag phase of 4 h and maximum growth occurred at 8 h. Further, the color of the medium had changed to yellow which might be due to the accumulation of *cis,cis*-muconic acid in the culture medium. An increase in the initial substrate concentration resulted in inhibitory effect on the actinomycete [19]. A marine cyanobacterium, *Phormidium valderiamum* BDU 30501 was able to grow up to a phenol concentration of 50 ppm and degraded 38 ppm within 7 days [20]. *Xanthobacter flavus* was able to tolerate phenol up to 1000 ppm and efficiently utilized and degraded it completely within 120 h when initial concentration was less than 600 ppm [21]. Similarly phenol degrading soil bacterium *Streptococcus epidermis* degraded phenol up to 200 ppm [22].

**Growth behavior of *Pseudomonas* sp. strain DS 002 in *p*-nitrophenol**

We found that the bacterium, *Pseudomonas* sp. strain DS 002 utilized *p*-nitrophenol also as sole source of carbon. The bacterium was studied at different concentrations of *p*-nitrophenol ranging from 0.5 to 2 mM. But among all concentrations, the *Pseudomonas* sp. strain DS 002 effectively grew on M9 media supplemented with 1 mM *p*-nitro phenol as sole source of carbon and energy. The growth behavior of *Pseudomonas* sp. strain DS 002 in *p*-nitrophenol maximum growth was observed at 36 h where the turbidity of the growth was found to be 0.34 at 600 nm (Figure 2A).

Several microorganisms are involved in the biodegradation of *p*-nitrophenol. The bacterium S*tenotrophomonas* utilized *p*-nitrophenol as the sole source of carbon, energy and degraded *p*-nitrophenol (500 ppm) with in 68 h [23]. Labana *et al.,* 2005 [24] reports on the isolation of *p*-nitrophenol degrading organisms.

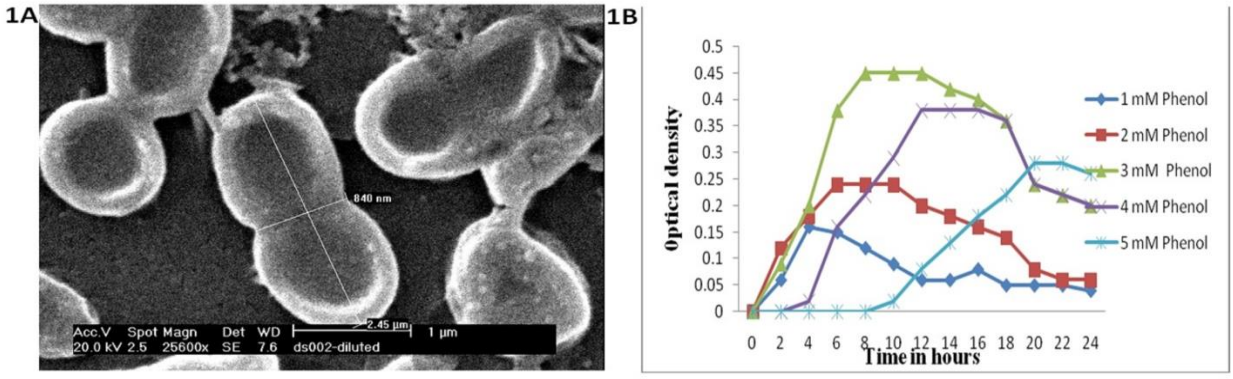


**Figure 1.** (A) Scanning Electron Microscopy image of *Pseudomonas* sp. Strain DS 002. (B).Effect of phenol on growth of *Pseudomonas* sp. strain DS 002.

**Effect of catechol on growth of *Pseudomonas* sp. strain DS 002**

*Cis,cis*-muconic acid is generally formed from the catechol its degradation following o*rtho*-cleavage pathway. In order to establish the formation of catechol from phenol, preliminary studies were conducted to assess the ability of *Pseudomona*s sp. DS 002 to grow on catechol as sole source of carbon. As expected catechol also served as the source of carbon and *Pseudomonas* sp. DS 002 grew effectively in M9 medium at different concentrations of catechol. The optimum concentration for the growth of *Pseudomonas* sp. strain DS 002 was found to be 0.5 mM catechol (Figure 2B). At this concentration it has attained maximum growth of 0.26 optical densities at 6 h. The growth pattern was shown in the Figure 2B. The color of the medium changed due to the formation of *cis,cis*-muconic acid when *Pseudomonas* sp. strain DS 002 was grown in catechol as sole source of carbon. Further, it also indicated that catechol and *cis,cis*-muconic acid may be the intermediates in the degradation of phenol. The strain *Pseudomonas putida* MTCC 1194 degraded catechol at concentration of 500 ppm over a period of three months [25]. The stain *Pseudomonas* sp. NGK-1 used 40 mM catechol as the sole source of energy and carbon in 72 h [26].

**Effect of *Cis,cis*-muconic acid and Hydromuconic semialdehyde (HMSA) on the growth of *Pseudomonas* sp. strain DS 002.**

*Cis,cis*-muconic acid and HMSA are the byproducts of catechol degradation via *ortho* and *meta* cleavage pathway. In order to know the pathway used by the *Pseudomonas* sp. strain DS 002, we have studied the growth behavior of the bacterium both in the *cis,cis*-muconic acid and HMSA. For this we have grown the *Pseudomonas* sp. strain DS 002 in the M9 medium having the *cis,cis*-muconic acid and HMSA as sole source of carbon at the concentration of 0.5 mM and monitored the growth for 24 hrs. The strain *Pseudomonas* sp. strain DS 002 degraded the *cis,cis*-muconic acid

and get maximum growth was observed at 4 h. We found the increase in turbidity due to growth of the bacterium only in the M9 medium having *cis,cis*-muconic acid as the sole source of carbon but not in HMSA. This implies that *cis,cis*-muconic acid is the intermediate in the degradation of catechol not the HMSA.

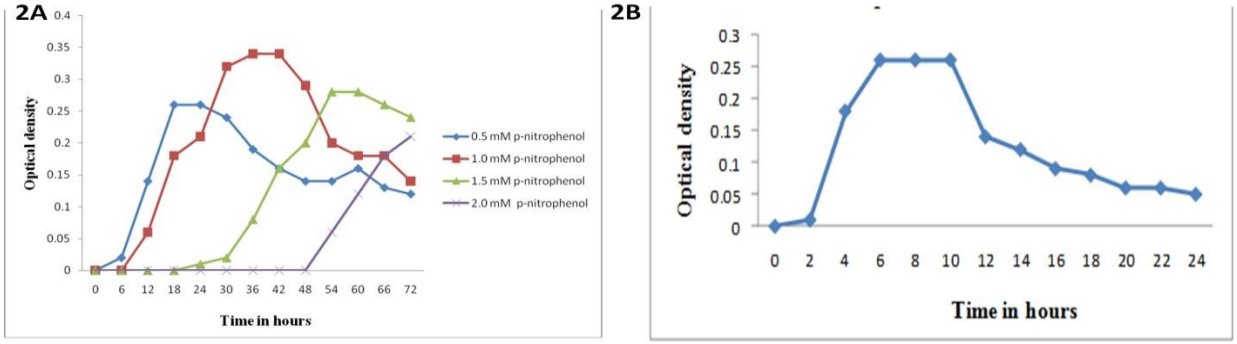


**Figure 2.** (A) Effect of *p*-nitrophenol (1 mM) on growth of *Pseudomonas* sp. Strain DS 002. (B) Effect of catechol (0.5 mM) on growth of *Pseudomonas* sp. strain DS 002.

**Catechol 1,2-dioxygenase and Catechol 2,3-dioxygenase activity**

Catechol 1,2-dioxygenase activity was studied in the cell free extract of the *Pseudomonas* sp. strain DS 002. The increase in the absorbance was observed with the formation of *cis,cis*-muconic acid with simultaneous decrease in the concentration of catechol. Further, there is no change or increased absorbance at 375 nm indicating that there is no formation of HMSA, which rules out the possibility of involvement of catechol 2,3-dioxygenase. This clearly demonstrated that *Pseudomonas* sp. strain DS 002 degrades catechol to *cis,cis*-muconic acid by using the enzyme catecol 1,2-dioxygenase and thereby activates complete degradation of catechol via *ortho* cleavage pathway.

**Oxygen up take studies in *Pseudomonas* sp. strain DS 002**

Microbial degradation of phenol is mediated by a number of monooxygenases and dioxygenases. The role of molecular oxygen is well established during the process of biodegradation. As evident from growth studies of *Pseudomonas* sp. *strain* DS 002 in phenol it is logical to expect consumption of molecular oxygen proportionate to the oxidative degradation of catechol. Therefore the resting cells of the bacterium prepared from the cultures grown in M9 medium supplemented phenol and glucose were used to study the rate of oxygen consumption in the presence of catechol. The resting cells grown in the medium supplemented with glucose did not consume any oxygen when catechol was supplemented to the resting cells. However the resting cells grown in phenol supplemented medium showed increased oxygen up take when catechol was added to the resting cells. When cells were grown under normal physiological conditions supplementing glucose as carbon source there was no oxygen consumption indicating any involvement of monooxygenase in the presence of glucose. On the contrary the oxygen consumption data on phenol grown cultures have shown higher levels of oxygen up take. The oxygen consumed by the cells on addition of catechol was calculated, which amounted to 48.4 nmol oxygen per minute per mg of dry cells. Mono and dioxygenase

enzymes are involved in phenol supplemented medium and utilized molecular oxygen for oxidative degradation of phenol which indicates that oxidative degradation of phenol utilized molecular oxygen.

**Analysis of intermediate metabolites of phenol biodegradation**

After isolating the data pertaining to the growth behavior of the strain of the medium an attempt was made to identify the catechol, the key intermediate in the phenol degradation pathway in the spent medium. Brown spots were detected in the lanes loaded with test sample extracted from the spent medium and *cis,cis*-muconic. No brown spot was observed in the lane loaded with the glucose grown cells (Figure 3A). It is assumed that the catabolite extracted from the spent medium of the phenol grown culture is *cis,cis*-muconic acid. These results further strengthen the data obtained from the TLC studies and prove that the *cis,cis*-muconic acid is the key catabolite formed during the degradation of phenol.

**Proposed bio-degradation pathway for phenol in *Pseudomonas* sp. strain DS 002**

Based on the growth behavior of the bacterium *Pseudomonas* sp. strain DS 002 phenol, *cis,cis*-muconic acid, catechol 1, 2–dioxgensae activity, oxygen uptake studies and TLC, we have concluded that the bacterium *Pseudomonas* sp. strain DS 002 used *ortho* cleavage pathway for the degradation of phenol and catechol 1,2-dioxygenase is the key enzyme involved in the degradation of catechol to *cis,cis*-muconic acid. Catechol 1,2-dioxygenase catalyses the intradiol cleavage (*ortho*-cleavage) of catechol and generates *cis,cis*-muconic acid is transformed to the instable enol-lactone, which is in turn hydrolyzed to oxoadipate. This dicarboxylic acid is activated by transfer to acetyl-CoA and succinate. These compounds were fed into the TCA cycle for further metabolism.

**Purification of Catechol 1,2-dioxygenase**

In order to carry out the enzyme activity, The cell free extract was made by freeze-thaw method and examined for the activity of catechol 1,2-dioxygenase which was found to be 3.318 µM/min. Total protein content was at crude stage is 1.892 mg/ml and specific activity is 1.754 µM/min/mg of protein. After ammonium sulfate precipitation and aliquots of the dialysis fraction were used for determining the activity of catechol 1,2-dioxygenase. This liquor mixture showed activity of 2.718 µM/min and 1.348 mg of total protein. The specific activity of catechol 1,2-dioxygenase is 2.017 µM/min/mg of protein. In this step 81% of protein was yield. The dialyzed sample was transferred to a packed column of DEAE- cellulose. The protein sample was eluted from the DEAE-cellulose column and the active fractions of enzyme show the activity of 2.465 µM/min and 0.980 mg of total proteins. In this step 74% protein was yield. The activity of the enzyme after final step of DEAE-cellulose chromatography purification was 1.640 µM/min and 0.480 mg/ml of protein, and the specific activity was 3.416 µM/min/mg. In this step 49% protein was yield. The eluted protein sample from gel filtration was subjected to SDS-PAGE and the molecular weight of catechol 1,2-dioxygenase of *Pseudomonas* sp. strain DS 002 was determined as approximately 32 kDa by SDS-

poly acrylamide gel electrophoresis (Figure 3B).

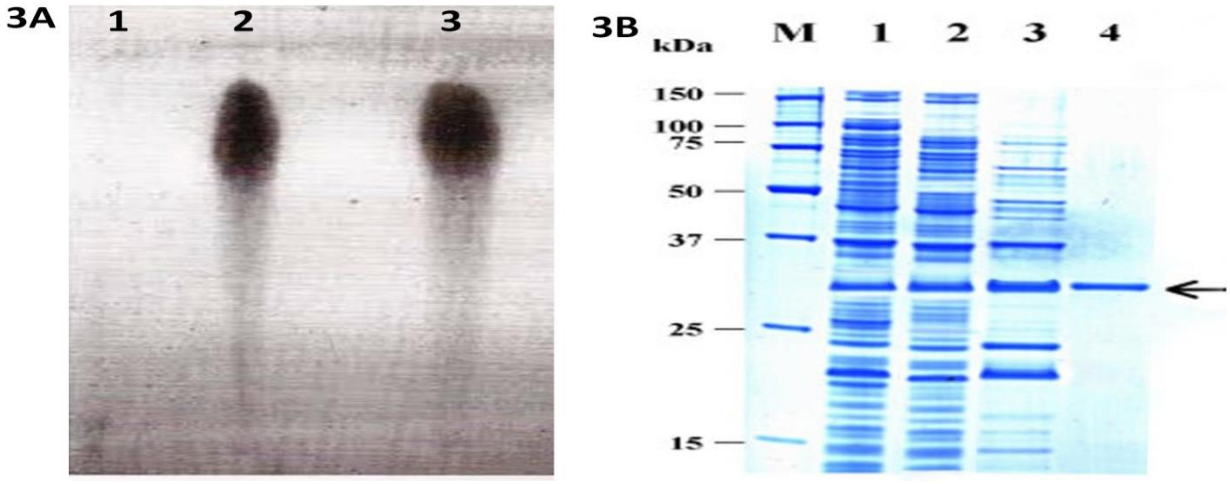


**Figure 3.** (A) TLC analysis of catabolytes estimated from the spent medium. Lane 1 and Lane 2 represent extract prepared from glucose and phenol grown cells. Lane 3 indicate standard *cis,cis*-muconic acid. (B) Purification of Catechol 1, 2-dioxygenase from *Pseudomonas* sp. strain DS 002. [M]. Molecular weight markers; [1]. Crude extract of *Pseudomonas* sp. strain DS 002; [2]. Ammonium sulphate precipitation elution; [3]. DEAE-Cellulose elution; [4]. Gel filtration elution.

**Effect of substrate concentration on Catechol 1,2-dioxygenaseactivity**

Effect of substrate concentration on catechol 1,2-dioxygenase in *Pseudomonas* sp. strain DS 002 was studied. Enzyme activity was measured at different concentrations of substrate i.e., 5, 25, 50, 75, 100, 125, 150, 175 and 200 µM. The enzyme activity was monitored by measuring the absorbance of *cis,cis*-muconic acid formed at 260 nm. The *Pseudomonas* sp strain DS 002 showed maximum activity of 1.580 µM/min at 100 µM concentration of the substrate. Hence it is calculated that the enzyme has an optimum substrate concentration of 100 µM. The catechol 1,2-dioxygenase isolated from *Ralstonia* Ba-0323 showed an optimum substrate concentration of 13 µM [27]. An intracellular catechol 1,2-dioxygenase of strain *Pseudomonas aeruginosa* showed optimum substrate concentration of 0.5 mM of catechol [28]. The catechol 1,2-dioxygenase of the *Rhodococcus* sp. NCIM 2891 showed maximum activity 10 mM of catechol [29].

**Effect of substrate specificity on Catechol 1,2-dioxygenaseactivity**

Substrate specificity of catechol 1,2-dioxygenase in *Pseudomonas* sp. strain DS 002 was studied. Results from the present study demonstrated that substrate specificity on catechol 1,2-dioxygenase in *Pseudomonas* sp. strain DS 002 varied as follows: 3-Methyl catechol (0.1 mM) 36%, 4-Methyl catechol (1.0 mM) 30%, 3-Isopropyl catechol (0.01mM) 63% whereas pyrogallol (0.1 mM) showed more than catechol 174% activity. Our results are in agreement with those reported in the literature. The substrate specificity of catechol 1,2-dioxygenase from *Acinetobacter* revealed 42% with 3-Methyl catechol, 36%, 4-Methyl catechol and 70% with 3-Iso-propyl catechol [30]. Benzoic acid was degraded by *Pseudomonas aeruginosa* by means of Catechol 1,2-dioxygenasewith

varying substrate specificity of 10% with 3-Methyl catechol, 43% with 4-Methyl catechol, 11% with 4-chloro catechol whereas 180% with pyrogallol [28].

**Effect of temperature on the Catechol 1,2-dioxygenase activity**

The effect of varying temperature on the catechol 1,2-dioxygenase was studied (Figure 4A)**.** There was an increase in the rate of oxidation of catechol with increase in the temperature up to 37°C. Further increase in the temperature resulted in decrease in rate of oxidation of catechol to *cis,cis*-muconic acid. The catechol 1,2-dioxygenase showed maximum activity at 37ºC and it has the stability in the range of 30 to 45ºC optimally. The catechol 1,2-dioxygenase exhibited maximum activity of 1.438 µM/ min at 37ºC. The optimum temperature was found to be 30 to 40ºC. The catechol 1,2-dioxygenase of purified protein in *Pseudomonas aeruginosa* showed optimum temperature at 40°C which was stable up to 40°C [28]. The catechol 1,2-dioxygenase of *Rhodococcus* sp AN-22 exhibited optimum temperature at 30°C [31]. *Pseudomonas fluorescence* used 4-chlorobenzoic acid as the sole source of carbon and the activity of catechol 1,2-dioxygenase was more at 25°C [32]. Catechol 1,2-dioxygenase from *Rhodococcus* sp. NCIM 2891 showed optimum temperature at 30°C [29].

**Effect of $p^H$ on the Catechol 1,2-dioxygenase activity**

The $p^H$ of a solution can profoundly influences the structure and activity of the enzymes. $P^H$ influences the state of ionization of acidic or basic amino acids in a protein. The effect of pH on the activity of catechol 1,2-dioxygenase in *Pseudomonas* sp. strain DS 002 was studied. The influence of $p^H$ on the enzyme activity was studied in a range of 3 to 9. The catechol 1,2-dioxygenase exhibited high activity in the range of pH 5 to 8. At $p^H$ 7 maximum activity was observed (Figure 4B). The 1, 2 dioxygenase from the *Pseudomonas* sp. strain DS 002 showed maximal activity with 1.564 µM/min at $p^H$ 7. The strain *Pseudomonas aeruginosa* has optimum pH of 7 to 9 [28]. In *Ralstonia* sp. Ba 0323 optimum $p^H$ is 8 [27] while benzoate degraded *Acinetobacter calcoaceticus* at an optimum $p^H$ of 7 [30], *Trichosporon cutaneum* showed maximal activity at $p^H$8.2 [33], *Rhodococcus erythropolis* showed optimum $p^H$ 7.5 [34]. The activity of 1,2-dioxygenase showed optimal $p^H$ of 9 to 9.5 in *Rhizobium leguminosarum* [35].

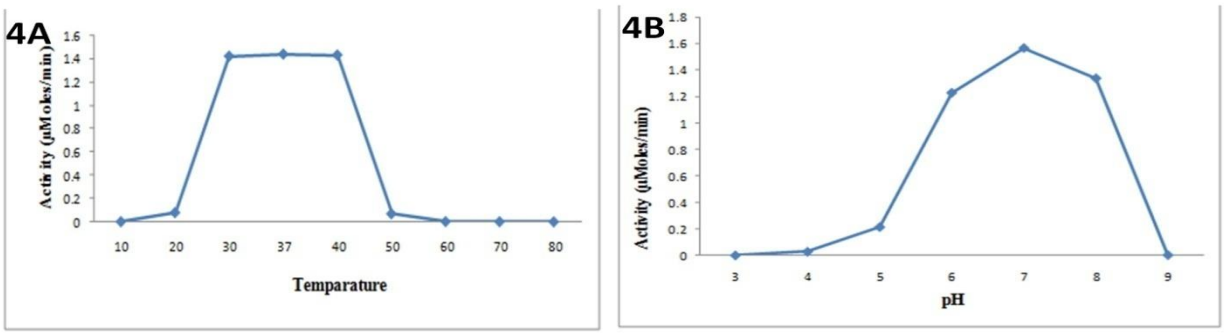


**Figure 4**. (A) Effect of temperature on Catechol 1, 2- dioxygenase. (B) Effect of $p^H$ on Catechol 1, 2-dioxygenase.

**Effect of Metal ions, Sulfhydryl and Chelating agents on Catechol 1,2-dioxygenase activity**

The effect of metal ions, sulfhydryl agents and chelating agents on enzyme activity was studied. A wide variety of metal ions are found in the environment and in the body. They serve many functions in proteins, the most important of which is the modification and the structural stability of the proteins. In our study the effect of silver nitrate, copper sulphate and ferrous sulphate on the activity of catechol 1,2-dioxygenase was studied. Silver nitrate inhibited 88%, copper sulphate 72% and ferrous sulphate 4% of the activity of catechol 1,2-dioxygenase from *Pseudomonas* sp. DS 002, whereas in *Acinetobacter calcoaceticus* silver nitrate inhibited completely, copper sulphate inhibited 91% and ferrous sulphate inhibited 12% of the activity of catechol 1,2-dioxygnease [30]. In benzoate degradation by *Ralstonia* sp Ba-0323, copper sulphate and silver nitrate inhibited completely and ferrous sulphate inhibited 66% of the activity of catechol 1,2-dioxygenase [27].In aniline degradation by *Rhodococcus* sp AN-22, silver nitrate inhibited 88%, copper sulphate 11% and ferrous sulphate 4% of the activity of catechol 1,2-dioxygenase whereas in L-Malate degrading *Rhodococcus* sp An-22, silver nitrate inhibited 77%, copper sulphate 13% and ferrous sulphate 2% of the activity of catechol 1,2-dioxygenase [31]. The effect of Tiron (0.1 mM) and α,α-Bipyridyl has no effect on catechol 1,2-dioxygenase of *Pseudomonas* sp. strain DS 002. Sulfhydryl agents like PCMB (*P*-chloromercuri benzoate), N-Ethyl malemide, DTNB (5,5-dithiosbis-2-nitrobenzoic acid) were used to study the effect of sulfhydryl agents on the activity of catechol 1,2-dioxygenase. Sulfhydryl compounds were not effective inhibitors of enzyme catechol 1,2-dioxygenase. Among sulfhydryl compounds only PCMB had 20% inhibitory effect on the activity of catechol 1,2-dioxygenase. N-Ethyl malemide (0.1 mM) inhibited 5% of the activity of catechol 1,2-dioxygenase and DTNB showed no effect on the activity. Our results are in agreement with those reported by Patel *et al*., (1976) [30] and Matsumara *et al*., (2004) [31], who studied the effect of sulfhydryl agents on the activity of catechol 1,2-dioxygenase from *Acenetobacter calcoaceticus* and *Pseudomonas aeruginosa* respectively. They reported that sulfhydryl compounds were not effective inhibitor of enzyme activity except PCMB. PCMB (0.5 mM) completely inhibited the activity of catechol 1,2-dioxygenase from *Ralstonia* sp. Ba-0323 and *Pseudomonas aeruginosa* [27] [28]. In our present study DTNB (5, 5 Dithobis-2-nitro benzoic acid) (0.1mM) has no effect on the activity of catechol 1,2-dioxygenase. DTNB (0.1 mM) has no effect on the activity of catechol 1,2-dioxygenase from *Acinetobacter calcoaceticus*[30] whereas DTNB inhibited 43% of the activity in *Ralstonia* species [27]. The effect of chelating agents such as EDTA, o-phenanthroline, Tiron, α,α-Bipyridyl on the activity of catechol 1,2-dioxygenase purified from *Pseudomoans* sp strain DS 002 was studied. EDTA (1.0 mM) inhibited 5% of the activity of enzyme in *Pseudomonas* sp. DS 002, whereas 4% activity in *Acinetobacter calcoaceticus* [30] and 33% activity in *Ralstonia* species [27]. In strain DS 002, aniline degrading *Rhodococcus* [31] and *Acinetobacter calcoaceticus* [30] *O*-Phenanthroline (1.0 mM) has no effect whereas activity increased by 5% in L-malate degrading

*Rhodococcus* [31].

## 4. CONCLUSION

The isolate was identified on the basis of morphological, physiological, biochemical studies it was identified as Pseudomonas species and designated as Pseudomonas sp DS 002. The strain DS 002 has effectively utilized phenol as the source of carbon. The strain DS 002 was grown at different concentration of phenol ranging from 1 mM to 5 mM and strain used catechol also as the source of carbon. Analysis of the intermediate metabolites of phenol degradation by TLC, enzymatic assay, oxygen uptake studies indicates that the strain DS 002 mineralized phenol via ortho-pathway. The catechol 1,2-dioxygenase exhibited maximum activity at 100 µM concentration of catechol. Its optimum pH and temperature were 7 and $37^0$C respectively.

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## CONFLICT OF INTEREST

Authors have no conflict of interest.

## REFERENCES

1. Kumar GN, Sumangala KB. Fungal degradation of Azo dye-red and optimization of physic-chemical parameters. ISCA Journal of Biological Sciences. 2012; 1(2): 17-24.
2. Pimentel D, Lewiton L. Pesticides amount applied and amount reaching pests. Bioscience. 1986; 36: 86-91.
3. Thomson J, Abbot DC. Pesticide Residues. Lecture Series No.3. The Royal Institute of Chemistry. 30. Russel Square, London. W.C.I. 1996; 34: 86-92.
4. Hill GA, Robinson CW. Substrate inhibition kinetics: Phenol degradation by *Pseudoonas pudita*. Biotechnology Bioengineering. 1975; 17: 1599-1615.
5. Li JK, Humphrey AE. Kinetics and fluorometric behaviour of phenol fermentation. Biotechnology letters. 1989; 11: 177-182.
6. Muftah H, EI-Naas, Shaheen A, AI-Muhtaseb, Makhlouf S. Biodegradation of phenol by *Pseudomonas putida* immobilized in polyvinyl alcohol (PVA) gel. Journal of HazardousMaterials. 2003; 164: 720-725.
7. Baroniya M, Baroniya SS, Jain M. Operation and Maintenance of water treatment plant at BNP Campus Dewas, India: A Case study. ISCA Journal of Biological Sciences. 2012; 1(1): 83-86.
8. Murhekar GH. Trace of metals contamination of surface water samples in and around Arot City in Maharastra, India. Research Journal of Recent Sciences. 2012; 1(7): 5-9.
9. Pawar MJ, Nimbalkar VB. Synthesis and phenol degradation activity of Zn and Cr doped $TiO_2$ Nanoparticles. Research Journal of Chemical Sciences**.** 2012; 2(1): 32-37.
10. Azin I, Katayon S. Degradation of phenol in wastewater using anolyte produced from

electrochemical generation of brine solution. Global Nest. International Journal. 2002; 4: 139-144.

11. Sally NJ. Extraction of phenol from Industrial Water Using Different solvents. Research Journal of Chemical Sciences**.** 2012; 2(4):1-12.
12. Pradhan N, Ingle AO. Degradation of phenol through ortho pathway by *Pseudomonas* sp.BC1. Indian Journal of Microbiology. 2003; 43: 267-269.
13. Chauhan A, Samata SK, Jain RK. Degradation of 4-nitro catecol by *Burkholderia cepacia:* a plasmid encoded novel pathway. Journal Applied Microbiology. 2000; 88: 764-772.
14. Begona PM, Hidalgo A, Serra LJ, Maria JL. Degradation of phenol by *Rhodococcus erythropolis* UPV-1 immobilized biolite in a packed-bed reactor. Journal of Biotechnology. 2002; 97:1-11.
15. Sivasubramanian K, Ravichadran S, Vigneswaran. Isolation of phenol degrading bacteria from decapods mangrove crabs. SOJ Microbial Infect Disease. 2016; 4(1):1-5.
16. Gopaul S, Compbell K, Robinson W, Imniss WE. Phenol degradation by psychotropic strain of *Pseudomonas putida*. Applied and Environmental Microbiology. 1991; 34: 539-543.
17. Hofrichter M, Gunther T. Metabolism of phenol, chloro and nitro phenols by *Pencillium* strain Bi 712 isolated from a contaminated soil. Biodegradation.1993; 3: 415-421.
18. Fillipowciz N, Momotko M, Boczkaj G, Pawlikowski T, Wanarska M, Cieslinski H. Isolation and Characterization of phenol- degrading Psychro tolerant Yeasts. Water Air Soil Pollution. 2017; 210-218.
19. Vidyavathi N, Srinikethan G, Charyulu NCLN. Biodegradation of phenol by *Nocordia hydrocarbonoxydans*. 36 th Annual Conference of AMI, Hissar, November 8-10.2000. 61: 233-239.
20. Shashirekha S, Uma L, Subramanian G. Phenol degradation by the marine *Cyanobactacterium Phormidium valderianum* BDU 30501. Journal Ind. Microbiology Biotechnology.1997; 19:130-133.
21. Lowry M, Nagamani A, Sreenivasulu K, Soligalla R. Isolation of phenol degrading soil bacterium *Xthantho bacterflavus.* Bioremediation. 2009. 13(1): 1-6.
22. Bhavana VM, Jalgaonwala RE, Pawar S, Morankar A. Isolation and characterization of phenol degrading bacteria from oil contaminated soil. InnovativeRomanian Food Biotechnology. 2010;7: 61-65.
23. Liu Z, Yang C, Qiao C. Biodegradation of *p*-nitrophenol and 4-chlorophenol by *Stretophotomonas* SP. FEMS Microbiology letters. 2007; 277.150-156.
24. Labana S, Singh OV, Basu A, Pandey G, Jain RK. A microcosm study on bioremediation of *p*-nitro phenol-contaminated soil using *Arthrobacter protophormiae* RKJ100. Environmental Biotechnology.2005; 68: 417-424.

25. Kumar A, Kumar S, Kumar S. Biodegradation kinetics of phenol and catechol using *Pseudomonas putida* MTCC 1194. Biochemical Engineering Journal. 2005; 2A2: 151-159.
26. Patil NK, Gouda U, Niazi J, Karegoudar H. Stable degradation by *Pseudomonas* sp. Strain NGK1 encapsulated in alginate and polyurethane foam. Indian Journal of Biotechnology**.** 2004; 3: 568-572.
27. Wang C, Takenaka S, Murakami S, Kenji AOKI. Production of catechol from Benzoate by the wild strain *Ralstonia* Species Ba-0323 and characterization of its catechol1,2-dioxygenase. Biotechnology Biochemistry.2001; 65(9):1957-1964.
28. Wang CL, You SL, Wang SL. Purification and characterization of a novel catechol 1,2-dioxygenase from *Pseudomonas aeruginosa* with benzoic acid as a carbon source. Process Biochemistry. 2006; 41: 1594-1601.
29. Naiem H, Nadaf, Jai S, Ghosh. Purification and characterization of catechol 1,2,-dioxygenase from Phodococccus sp. NCIM 2891. Research Journal of Environmental and Earth Sciences. 2011; 3(5): 608-613.
30. Patel RN, Hou CT, Felix A, Lillard MO. Catechol 1,2-dioxygenase from *Acinetobacter calcoaceticus*: Purification and properties. Journal of Bacteriology.1976; 127:536-544.
31. Matsumura E, Ooi S, Murakami S, Takenaka S, Aoki K. Constitutive synthesis, purification and characterization of *catechol 1, 2 dioxygenase* from the Aniline-Assilmilating bacterium *Rhodococcus* sp. AN-22. Journal of Bioscience andBioengineering.2004; 98: 71-76.
32. Saxena P, Thakur IS. Purification and characterization of catechol 1,2-dioxygenase of *Pseudomonas fluorescens* for degradation of 4-chloro benzoic acid. IndianJournal of Biotechnology. 2005; 4:134-138.
33. Varga JM, Neujahr Y. Purification and properties of *catechol* 1, 2 *dioxygenase* from *Trichosporon cutaneum*. European Journal of Biochemistry. 1970;12: 427-434.
34. Aoki K, Konohana T, Shinke R, Nishira H. Purification and characterization of catechol 1,2-dioxygenase from aniline-assimilating *Rhodococcus erythropolis* AN-13. Agriculture Biology Chemistry. 1984; 48: 2085-2087.
35. Chen YP, Charles R, Lovell. Purification and properties of catechol 1, 2 dioxygenase from *Rhizobium leguminosa rumbiovarviceae* USDA 2370. Applied and Environmental Microbiology.26: 1990; 1971-1973.