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Biodegradation of Phenol by Isolated Bacterial Strain *Psuedomonas* spp. from Gola river water of Nainital

Ramendra Nautiyal [1,2], Prashant Singh [2] and Sanjay Gupta [1]*

[1] Department of Biochemistry and Biotechnology, SBSPGI, Balawala, Dehradun, India
[2] Department of Chemistry, DAV (PG) College, Dehradun, India

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Abstract: The biodegradation process for phenol was investigated using an isolated single bacterial culture of *Psueudomonas spp.* by serial dilution of river water and soil samples procured from the river bank of river Gola, Nainital. The *Psuedomonas spp.* was able to reduced 80-85 % pollution load within 48 hrs under aerobic condition in terms of Chemical Oxygen Demand (COD) in River Water containing Phenol (RWP). These strains were examined for their ability to degrade phenol by plate assay method by varying phenol concentration (1 mM-10 mM) using Phenol-MSM media. The mode of ring cleavage was being carried-out via ortho-cleavage pathway i.e., phenol hydroxylase was responsible for the high tolerance to high phenol concentration as correlated by *Psueudomonas spp.* showing, the maximum activity of *phenol hydroxylase.* The influence of four process parameters studied temperature (20-60^{o}C), pH (4-9), substrate (1-10 mM) and agitation speed (30-150 rpm) influenced the rate of biodegradation extent. Optimum pH for phenol biodegradation was observed at 7.5, which was almost stable at increasing pH range up to 8.0. The effect of temperature on the activity of enzyme was significant and maximum activity lies within the range of 35-45^{o}C at static condition at an optimum substrate concentration of (6-8 mM). The enzyme activity remained fairly good at 55^{o}C i.e., enzyme is stable at high temperature.

Key words: Biodegradation, *Psuedomonas* spp. phenol, organic pollutants.

Introduction

The toxic pollutants phenolics, cyanides, thiocyanides, formaldehydes, pesticides and heavy metals are some of the most common potentially dangerous substances that are appearing in nature due to effluents of heavy industries [1,2]. These effluents are typically toxic, colored and turbid with high amount of suspended solids, organic or inorganic in nature. Although some of them can be readily treated, while others are recalcitrant or xenobiotic compounds being poorly degradable, accumulate in soil and water and finally cause highly toxic effects on human beings, animals and plants through biomagnifications. Therefore, to protect the environment from such toxic materials, the waste water and industrial effluents must be suitably treated before discharge for sustainable development [3,4]. Phenols and its derivatives are highly persistent, bioaccumulative and toxic aromatic compounds of serious environmental concern because of their widespread use, toxicity for aquatic species, and occurrence throughout the environment [5,6,7]. Polybutylene terephalate (PBT) compounds are not readily broken down and easily metabolized in environment. They may accumulate in human body and food chains through their consumption or uptake [8,9]. The toxicity and environmental recalcitrance of these compounds

*Corresponding author (Sanjay Gupta)
E-mail: < sanjay_gupta9999@rediffmail.com >

have prompted studies on their degradation by microorganisms [10,11,12]. The microbial degradation of phenolic compounds is often hampered by toxicity exerted by high concentration of these compounds. The degradation of phenols and its derivatives by bacteria, fungi and yeast is well reported and extensively studied. Numerous bacteria are known to degrade Xenobiotics/phenolic compounds were isolated most frequently from the soils, sediments of various sludges which are contaminated by the xenobiotics of interest, mainly belong to of genera *Alcaligenes, Arthrobacter Pseudomonas, Rhodococcus, Bacillus, Klebsiella, Ralstonia and Acinetobacter* [13,14,15,16]. The key enzymes necessary for xenobiotics degradation did not evolve de novo as a reaction to anthropogenic activities during the last several decades, but existed in nature for a considerable longer time. Their original substrates seem to be natural substances with structural attributes similar to present day xenobiotics. In case of phenolic compounds, such substances are various plant products including degradation products of lignin, flavanoids, some alkanoids, plant pigments, isoprenoids and many others [17,18,19]. In the present study bacterial strains were isolated from the soil and water of Gola river, Nainital, Uttarakhand. The strains that are capable of degrading phenol and substituted phenol pollutants to a considerable extent (80-90 %) upto 10-15 mM concentration in 24-48 h of culture incubation were isolated and studied for various parameters both physical and chemical, contributing towards the efficient degrading process.

Material and methods

Sample collection area and its preservation

Water samples of river Gola were collected in Tarson bottles previously rinsed with non-ionic detergent, rinsed with tap water and later leached with 10 % nitric acid for 24 hours and finally rinsed with deionised water prior to use. This process has importance because certain metal ions like chromium, cadmium, lead are subject to loss or adsorption or ion exchange with walls of container. The soil samples were collected in the sterilized zip lock polybags to prevent environmental contamination. All the samples after maintaining in cold chain were brought to the laboratory to store in sampling box at 4°C.

Culture media, strain isolation and characterization

To prepare the suspensions of bacteria, 10 g of aseptically weighed soil was added to 100 ml of suspension solution (Tween-80 2.0 gl^{-1}; carboxymethylcellulose CMC 2.5gl^{-1}; NaCl 5.0 gl^{-1}) and the mixture was vigorously shaken for 15 min. Following a short period of sedimentation (3 min), the water phase was recovered for direct plating and preparation of appropriate dilution, 100 µl of sample was spread onto the Nutrient Agar (NA) and Tris Minimal Salt (TSM) Media plates and incubated at 37°C for 24 hrs [20], so that every colony represents, a pure culture which were plated subsequently on solidified media. In case of river water sample direct plating was carried out on solidified media. The isolates were identified by biochemical characterization and bacterial growth kinetics.

Media for screening of phenol degrading microorganisms

For screening the phenol degrading capability of isolated microorganisms [21,22], Phenol Minimal Salt Agar Media was used. Phenol-MSM medium contained (gl^{-1}) K_2HPO_4 3.0 ; KH_2PO_4 2.0 ; Fe $SO_4.7H_2O$ 0.005 ; Mg SO_4 0.05 ; NH_4Cl 0.5 ; bromothymol blue 0.06; trace element solution 1.0 ml. Phenol was added before solidifying to mineral agar to obtain 1 mM to 10 mM concentration. Cultivation was carried for 3 days at 37°C. Phenotypically different colonies obtained from the plates were transferred to fresh mineral agar plates with and without phenol to eliminate autotrophs and agar utilizing bacteria. Utilization of phenol is accompanied by a decrease in pH and thus a color change from green to yellow of the bromothymol blue present in the media. The procedure was repeated and only isolates exhibiting pronounced growth on phenol were stored for further characterization. Further, the shake flask studies were conducted using wastewater supplemented with minimal mineral media. The organism was used for subsequent treatment and optimization studies.

Characterization of bacterial isolates

Morphological and Biochemical characterization of bacterial isolates was performed according to Bergey's Manual of Determinative Bacteriology [23].

Optimization of growth factors

All the major factors influencing growth in presence of phenol in Phenol-MSM such as the incubation period, substrate concentration, pH and temperature were optimized by performing experiments in 100 ml flasks in shaking condition at 150 rpm. Total and residual phenol concentration under aerobic conditions was evaluated in liquid mineral media with phenol as substrate by decrease in absorbance at 270 nm [24]. Further, the phenol was also estimated by 4-amino antipyrine method as described in APHA[25].

Elucidation of mode of ring cleavage of phenol

After 24-48 h of culture incubation/growth in Phenol-MSM media, 0.5 M of catechol was sprayed on to the colonies. The formation of an intense yellow color indicated the presence of catechol 2,3-dioxygenase, indicating that phenol is degraded via meta-cleavage pathway. Absence of yellow color possibly indicates the ortho-cleavage pathway. Further for detection of an ortho-cleavage mechanism the Rothera reaction was carried out[22].

Enzyme assay for phenol hydroxylase activity

Phenol hydroxylase is the main catalytic enzyme for the ortho-hydroxylation of phenol to catechol. To study the kinetics of phenol hydroxylase, standardization of the spectrophotometric enzyme assay has been performed. 24 h culture samples were centrifuged at 8000 rpm for 10 min. The resulting pellets were washed twice with the extraction buffers (0.05 M phosphate buffer, pH 7.6, containing 1mM 2-mercaptoethanol, 0.1 mM EDTA, 0.001 mM FAD) and subsequently the cells were disrupted with glass beads (0.45 mm diameter). Unbroken cells and cell debris were removed by centrifugation at 12000xg for 30 min at 4^{o}C. All the supernatant were used without further treatment to detect enzyme activity. The assay for phenol hydroxylase (EC 1.14.13.7) was conducted spectro-photometrically by monitoring the absorbance decrease at 340 nm [18]. Spectrophotometric measurement were recorded every 15 sec for 2 min. at room temperature. One enzyme unit was defined as the amount of enzyme that catalyzes the consumption of 1 micromole co-substrate or formation of 1 micromole product per minute.

Results and discussion

Isolation and test of phenol tolerance

A total of 30 bacterial isolates were able to grow on Phenol-MSM media using 1-10 mM phenol as a sole carbon source from soil and river water of Gola, Nainital. After the disappearance of phenol, an aliquot was transferred to fresh medium. After several transfers an aliquot was spread onto the Phenol-MSM media agar. The disappearance of phenol from the plated media, which was indicated by color change of bromothymol blue from green to yellow 10 colonies were selected for further screening (Fig. 1). Among the 30 isolates, six pure cultures designated as B1-B6 were finally selected for the further studies based on phenol tolerance in assays utilizing 1-10 mM phenol (Table 1). Other isolates were discarded based on their poor growth and similar appearance on Phenol-MSM media. Based on the microscopic morphological and biochemical characterization as per the Bergey's manual of determinative bacteriology, the strains were identified as belonging to the genus *Pseudomonas* and *Bacillus sp.* and were designated as B1: *Bacillus spp RN1*, B2: *Bacillus spp RN2*, B3: *Bacillus spp. RN3*, B4: *Micrococcus spp.*, B5: *Psueudomonas spp. RN1*, B6: *Psuedomonas spp. RN2.* No molecular phylogeny method was followed to confirm the species. Further, to determine the growth and multiplication of bacterial cells, the highest cell numbers were observed after 24 h of incubation and this corresponds to the late log phase/early stationary phase of *Psuedomonas spp. RN2* growth cycle. It has been observed that maximum degradation capability occurs in the initial stage of the stationary phase which corresponds to the maximum numbers of the viable cells of

Table 1. Growth characteristics of bacterial isolates on phenol as sole source of carbon

Strains	1 mM	2 mM	3 mM	4 mM	5 mM	6 mM	7 mM	8 mM	9 mM	10 mM
Bacillus spp. RN1 B1	‡	‡	‡	+	+	+	+	-	-	-
Bacillus spp. RN2 B2	‡	‡	‡	‡	‡	‡	‡	‡	+	-
Bacillus spp. RN3 B3	‡	‡	‡	‡	‡	+	+	+	+	+
Micrococcus spp. B4	‡	‡	‡	‡	‡	‡	‡	+	+	-
Psuedomonas spp. RN1 B5	‡	‡	‡	‡	‡	‡	‡	‡	-	-
Psuedomonas spp. RN2 B6	‡	‡	‡	‡	‡	‡	‡	‡	‡	‡

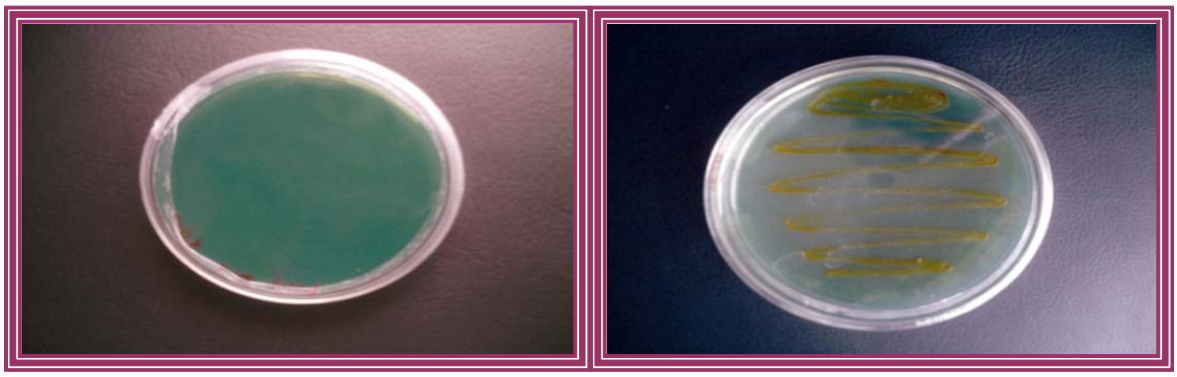
++++ = Luxurious growth
+++ = good growth
++ = fair growth
+ = poor growth
- = no growth

bacteria in growth media. Fig. 2 showed the adaptation period of 15 hours for the bacterial strain *Psuedomonas spp. RN2* with the river water supplemented media, followed by a rapid growth period of 15-22 hours and consequently showed the decline phase after 35 hours of culture growth. The results showed a typical bacterial growth curve corresponding to the physiology of the bacterial growth kinetics. The major factors incubation period, substrate concentration, pH and temperature that influence growth were further optimize

Optimization of different growth parameters of bacterial isolate (*Psuedomonas spp. RN2*)

On the basis of morphological and biochemical characterization, the strains were identified and belong to three major genus *Bacillus spp.*, *Pseudomonas spp.* and *Micrococcus spp.*[11]. The strains were further characterized and it was observed among the six selected phenol degraders B6: *Psuedomonas spp. RN2* showed the luxuriant growth up to 8 mM concentration of phenol in minimal media (Table 1) Therefore, *Psuedomonas spp. RN2* was further subjected to the optimization studies under different nutritional parameters to observe the enhanced reduction capability. In order to find out the optimum pH for the effective degradation of river water by *Psuedomonas spp. RN2*, experiment was performed at different pH and the results obtained are shown in Table 2. Maximum reduction (87 %) capability was observed in terms of all pollutant parameters at pH 7.0-80. As the treatment process was drifted towards the alkaline conditions at pH 8.0 and 9.0, the sharp decrease in the reduction capabilities of the bacterial strain was observed. The obtained results showed that pH 7.5 was desirable for the maximum degradation capabilities through the above tested strain of *Psuedomonas spp. RN2*. Therefore, the further studies were carried out at pH of 7.5.

The culture transfer technique was selected for microbial communities that were adapted to a specific temperature as well as the feast-famine growth conditions imposed and corresponding results are reported in Table 3. The impact of temperature on high pollution load during the biological treatment of the Gola river water was deter-



Control **Positive**

Fig 1. Screening of bacterial strains degrading phenol indicated by yellow colored colonies on the Phenol-minimal salt media

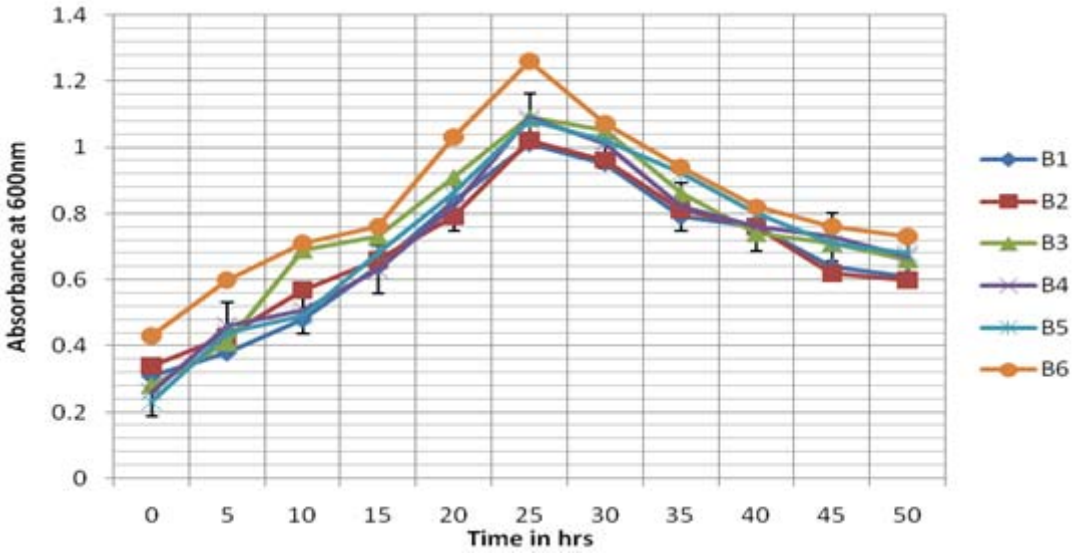


Fig 2. Growth kinetics study of different isolated bacterial culture in Phenol Minimal salt media (Phenol MSM) B1: *Bacillus spp.RN1*, B2: *Bacillus spp.RN2*, B3: *Bacillus spp.RN3*, B4: *Micrococcus spp.*, B5: *Psueudomonas spp. RN1*, B6: *Psuedomonas* spp. *RN2*

mined for a culture transfers. The efficiency of pollution load removal were best at 35°C (91 %), however pollution removal declined as temperature was incrementally increased to 45°C (65 %). Very less or no bacterial growth, and thus very less and no pollution removal, occurred at temperature higher than 55°C.

Effect of shaking and static condition at different incubation time period (0-45 hrs) and aeration conditions ranging from 0, 50, 100 and 150 rpm were used to measure its effect on reduction capabilities of the tested strain i.e. *Psuedomonas spp.* RN2 for all the five selected parameters viz. TDS, electrical conductivity, COD, BOD and phenols It was observed that under static condition and at 35 hrs of culture incubation showed maximum degradation efficiency of 80-89 % was achieved. At the higher agitation speed condition, the lower reduction capability of the strain was observed as shown in Fig 3.

Elucidation of mode of ring cleavage of phenol

Absence of yellow color on spraying catechol/ resorcinol solution on phenol minimal salt agar media with growth of the bacterial isolates, indicated the ortho-cleavage pathway for degradation of phenol and the enzymes for phenol deg-

Table 2. Percent reduction of different parameters for river water treatment by *Psuedomonas spp. RN2* at different pH

	% Reduction				
pH	TDS	EC	COD	BOD	Phenols
5.0	28	27	33	38	37
5.5	39	38	44	54	49
6.0	61	60	64	59	71
6.5	68	70	68	75	78
7.0	74	77	84	80	84
7.5	85	76	93	90	87
8.0	80	75	87	86	81
8.5	32	33	45	39	42
9.0	-	-	13	10	22

Table 3. Percent reduction of different parameters for river water treatment by *Psuedomonas spp. RN2* at different temperature

	% Reduction				
Temperature(°C)	TDS	EC	COD	BOD	Phenols
25	38	48.3	51	48	54
30	60	58.4	66	54	62
35	86	85	90	88	91
40	86	85	80	76	83
45	68	69	69	52	65
50	52	62	59	50	58
55	44	45	47	34	43

radation pathway were not constitutively expressed. It may be correlated that phenol degradation by ortho pathway is specified by plasmid in these species [22].

Phenol hydroxylase activity

The tested strains of *Pseudomonas putida* showed the maximum activity of *phenol hydroxylase* was observed at pH 7.5, which was almost stable in the pH range of 7.0-8.0, which shows that this strain can be used to treat the waste water with mild alkalinity. Effect of temperature on the activity of enzyme was quite significant and lies within the range of 35-45°C, with a maximum activity at 40°C become non significant as the temperature rises above 55°C i.e., enzyme is not stable at high temperature. However, the activity was quite stable over the broad concentration 1-10 mM phenol concentration, which indicates that these enzymes can remain active at wider range of phenol concentration and this property of these enzymes can be utilize to treat various industrial waste containing varying amount of phenol as pollutant.

A large number of microorganisms including bacteria, fungi, and algae [26] are capable of degrading phenol. The biodegradation of phenol and its derivatives by bacteria has been extensively studied and a large number of phenol-degrading bacteria have been isolated and characterized at the physiological and genetic levels [27-32] Pure and mixed cultures of the *Pseudomonas* genus are the most commonly utilized biomass for the biodegradation of phenols 50 and they are believed to have good potential for different biotechnological applications. Specifically, *Pseudomonas*

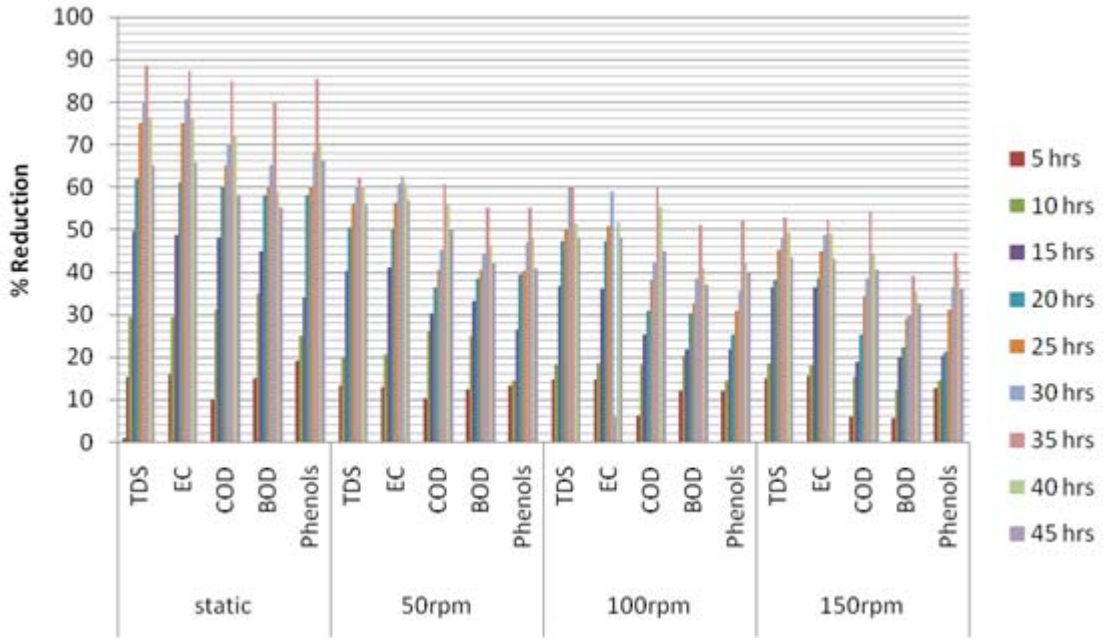


Figure 3. Time Course Study of static and shaking condition for biodegration of pollutants in Gola river water by *Psuedomonas* spp. *RN2*

putida has been commonly used for the biodegradation of phenol due to its high removal efficiency [33,34]. Responses of *P. putida* to chemical stresses have indicated that its cells could use diverse protective mechanisms for survival in various extreme environments [35]. The present study could help in synthesizing new bacterial strain with enhanced degradation capability and improved tolerance to toxic pollutants.

Conclusion

Biodegradation of phenols can be mediated by bacteria, fungi, and algae. It involves the breakdown of organic compounds through biotransformation into less complex metabolites, and through mineralization into inorganic minerals, H2O, CO2 (aerobic), or CH4 (anaerobic). In essence, microbial metabolism is a process of energy conversion and it is governed by enzymatic mechanisms, where reaction intermediates play a vital role. Biodegradation is a multifaceted process that incorporates several important factors and not only the intrinsic biodegradation potential in estimating the feasibility of biodegradation or bioremediation at a site. These factors may include bioavailability, temperature, pH, oxygen availability, substrate concentration, and biomass abundance as well as the physical properties of the contaminants. Thus, to devise a bioremediation system, all these factors are to be counted for, with particular consideration of the substrate concentration because it inhibits the growth of the organism at higher concentrations. Both bacteria and fungi have been extensively studied for their ability to degrade phenolic compounds, but the work in the algal group is scant. Most of research work has been focused on the bacterial species of *Pseudomonas* genus because of their proved high phenol removal efficiencies. The present research work has addressed issue including research on bacterial cultures of microorganism and studies with high initial phenol concentrations In this regard the strains were isolated by serial dilution of river water and soil samples procured from the river bank of river Gola, Nainital. These strains were examined for their ability to degrade phenol by plate assay method by varying phenol concentration (1 mM-10 mM) using Phenol-MSM media. Among the isolated strains, *Psueudomonas spp. RN2* showed, the maximum potential for degrading phenol and phenol substituted compounds. The influence of four process parameters such as temperature (20-60^{o}C), pH (4-9), substrate (1-10 mM) and agitation speed (30-150 rpm) influenced the

rate of biodegradation extent. Optimum pH for phenol biodegradation was observed at 7.5, which was almost stable at increasing pH range upto 8.0, which shows that this strain can be used to treat the water even at low alkalinity. Effect of temperature on the activity of enzyme was significant and maximum activity lies within the range of 35-45^{o}C at static condition at an optimum substrate concentration of (6-8 mM). The enzyme activity remained fairly good at 55^{o}C i.e., enzyme is stable at high temperature. This pattern of activity with temperature can be used to treat the waste water, solid waste with variable temperature and substrate range. Further, the mode of ring cleavage was determined by spraying 0.5 catechol solution on the bacterial colonies, absence of yellow color indicated that degradation was being carried-out via ortho-cleavage pathway i.e., phenol hydroxylase was responsible for the high tolerance to high phenol concentration.

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