ISSN Online: 2349-7785

Determination of Location of Phenol Resistant Gene in Phenol Degrading Bacterial Strains Expressing Phenol Hydroxylase Activity

Nidhi Srivastava [1], Vikash Singh Jadon [2], Rajender Singh Rana [2,3], Prashant Singh [3] and Sanjay Gupta [2]*

[1] Department of Microbiology, Sardar Bhagwan Singh P.G. Institute of Biomedical Sciences & Research, Balawala, Dehradun, India
[2] Department of Biotechnology & Biochemistry, Sardar Bhagwan Singh P.G. Institute of Biomedical Sciences & Research, Balawala, Dehradun, India
[3] Department of Chemistry DAV (PG) College, Dehradun, India

Received 15 October 2013; accepted in revised form 10 December 2013

Abstract: Phenols in waste water consist of variety of hydroxybenzene and substituted hydroxybenzene. These phenolic compounds in waste water get mixed up with river, oceans and also contaminate the soil which is very dangerous for flora and fauna even at low concentration. Nowadays degradation of such xenobiotic compounds by microbial activity is considered as the widely accepted process of waste treatment and environment pollution control. This treated waste water can then be used for irrigation purposes in agriculture as this treated waste water will have negligible toxic pollutant and deemed fit as per the norms of CPCB. So, the present study has been done to isolate the phenol degrading bacterial strains expressing Phenol hydroxylase activity on Minimal Salt Media (MSM), Different growth factors were optimized. Three strains NY-1, NS-1 & SP-2 were found to be resistant to phenol concentration up to the range of 5-7 mM and degradation was found to be in the range of 80-90 %. Mode of ring cleavage was found to be ortho-cleavage which is carried out by the enzyme Phenol hydroxylase. Therefore to determine location of phenol resistant gene acridine orange mediated plasmid curing was done taking antibiotic resistant property of the bacterial strain of interest as selectable marker.

The concentration of acridine orange at which the plasmid of the bacterial isolates were cured was found to be in the range of 25-40 μg/ml, above which the concentration of acridine orange was found to be lethal to bacterial growth. At 40 μg/ml, plasmids of all the three bacterial isolates got cured completely. The cured bacterial colonies were screened using the replica plate method. The colonies that showed no growth on the replica plate containing appropriate antibiotic salt as compared with the master plate were further screened for their phenol degrading capability. These colonies were inoculated in the Minimal Salt Media and were kept for incubation at 37°C for 24-72 h under shaking condition. Phenol degradation was almost negligible, which indicated that genes encoding for enzyme Phenol hydroxylase was plasmid borne.

Key words: Phenols, bioremediation, Phenol Hydroxylase, xenobiotic.

Introduction

Phenol also known under an older of carbolic acid is a toxic, colorless, crystalline solid with a sweet tarry odor. Phenol or hydroxybenzene has been categorized under xenobiotic compounds which are man made molecules foreign to life and should have never been encountered by bacterial population before their introduction by man.

Since phenol is an aromatic compound and

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

these compounds exhibit toxicity, mutagenic and carcinogenic properties. Phenols in waste water consist of variety of hydroxybenzene and substituted hydroxybenzene. These phenolic compounds in waste water get mixed up with rivers, oceans and also contaminate the soil which is very dangerous for flora and fauna even at low concentrations. One of the most promising methods is the application of hydrocarbon degrading bacteria to clean up the contaminated sites. The largest phenol tolerance has been observed in species of *Pseudomonas, Bacillus, Klebsiella,* and *Acinetobacter*. Mutzel *et al*., reported that a number of *thermophillic Bacillus* species, isolated from mud sample from a hot spring in Iceland, are capable of degrading phenols [1]. *Klebsiella oxytoca* strain grows on phenol as the only source of carbon and energy. *Pseudomonas putrefacien, Pseudomonas cepacia* and *Pseudomonas acidovorans* were capable of degrading both hydrocarbons as indicated by the yellow color formation (positive reaction) [2]. The enhancement of 2-chlorophenol degradation can be done by mixing the microbial community augmented with *Pseudomonas putida, Pseudomonas alcaligens,* and *Pseudomonas mendocina* by root zone process [3]. *P. putida* P106 and *Rhodococcus erythropolis* NY05 had shown significant positive chemo-static response toward Biphenyls. *Pseudomonas* species B4 had also shown chemo-attraction toward Polychlorinated biphenyls [4,5,6].

Phenol contaminated soil have shown that bacteria can adapt to ambient phenol concentration but increasing phenol concentration appears to decrease overall phenol biodegradation[7]. The enzymes involved in phenol biodegradation are phenol hydroxylase and catechol 1,2-dioxygenase which have broad specificity. The regulation of the synthesis of these two enzymes has been studied in bacteria, in which the genes coding for these enzymes are located on plasmids or on the chromosome of the microorganisms [8]. The strains belonging to genera *Staphylococcus, Corynebacterium, Bacillus* and *Proteus* were found resistant to 15 mM phenol. Location of phenol resistant gene was determined by acridine orange mediated plasmid curing. Interestingly, all the resistant isolates lost the characteristic (to resist phenol) after curing, thereby indicating the plasmid genes responsible for this property. Plasmid DNA isolated from the uncured strains was transferred to the cured competent recipient cells. Stable intrageneric transfer of phenol resistant plasmid gene(s) was observed by Munazza Ajaz *et al*.[9].

Objective

Sources of bacterial strains for biodegradation of xenobiotics are most frequently soil sediments of various sludges which are contaminated by the xenobiotics of interest. So, the present study is aimed to isolate the phenol degrading bacterial strains for the potential of excretion of extracellular *phenol hydroxylase* from different phenol contaminated sources *viz.,* compost oil contaminated soils and laundry effluents, to identify and characterize these bacterial isolates, to optimize the parameters influencing phenol degradation, to estimate the residual phenol concentration from 0-72 hrs of incubation, to elucidate the mode of ring cleavage of phenol, to determine the antibiotic sensitivity in isolated strains using antibiotic-disc method and to determine fate of phenol resistant gene by acridine orange mediated plasmid curing using antibiotic resistant property as a selectable marker.

Materials and methods

Isolation of microbial strains from soil samples

Isolation of pure colonies was observed by serial dilution of soil sample from compost and laundry effluent to thin out the population sufficiently and 100 µl of sample was spread onto the Nutrient agar plates, so that every colony represents a pure culture.

Media for screening of phenol degrading microorganisms

For screening the phenol degrading capability of isolated microorganisms, Phenol-Minimal Salt Media (Phenol-MSM) was used. Phenol-MSM medium contained:

K_2HPO_4 (30.0 g/l), KH_2PO_4 (20.0 g/l), $FeSO_4.7H_2O$ (0.005 g/l), $MgSO_4$ (0.05 g/l), NH_4Cl (0.5 g/l). Media pH was adjusted to 7.2.

Confirmatory test

The best colonies showing growth in phenol minimal salt media were named as NS-1, NY-1, SP-2, NY-5 and NY-6. These five strains were further screened to asses their capability of utilizing the various carbon sources. The four solid mediums- A [Agar + SDW], B [NAM], C [MSM+Phenol+SDW] & D [MSM+SDW] were designed with verified constituents, to assess the capability of bacterial isolates utilizing the various carbon sources.

Characterization of bacterial isolates

Morphological and Biochemical characterization of bacterial isolates was performed according to K.R. Aneja [10].

Optimization of growth factors

All the major factors influencing growth such as the incubation period, substrate concentration, pH and temperature were optimized by performing experiments in 100 ml flasks in shaking condition at 140 rpm and residual phenol concentration was estimated by Folin-Ciocalteau reagent.

Elucidation of mode of ring cleavage of phenol

After incubation of 24-48 h of inoculated Phenol-MSM media, 0.5M of catechol/ resorcinol was sprayed on to the colonies. The formation of an intense yellow color indicated the presence of catechol 1, 2-dioxygenase, indicating that phenol is degraded via meta-cleavage pathway. Absence of yellow color possibly indicates the ortho-cleavage pathway [11].

Determination of antibiotic sensitivity of strains

To determine the sensitivity of test organisms against some disc antibiotics. Disc diffusion method was used for this purpose [10].

Plasmid curing

For determining whether the gene encoding for the enzyme for phenol biodegradation is plasmid DNA or chromosomal DNA borne, plasmid curing using *acridine orange* is the best suited method that can be used easily in the laboratory. Plasmid curing was done according to the protocol available in the standard book *Samrook & Maniatis* [12].

Results and discussion

Isolation of bacterial strains

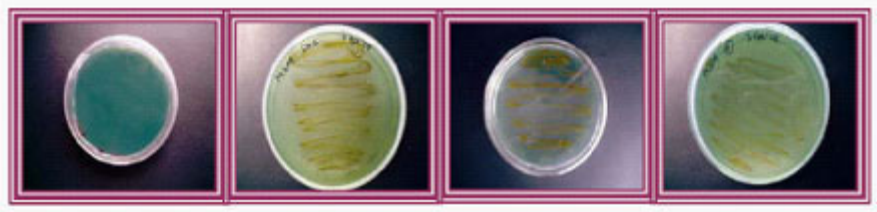
Different samples collected from different sources that are free of industrial pollution were serially diluted and spread on NAM, about 190 isolates, showing growth on NAM, were isolated and purified on respective cultures.

Screening of phenol degrading isolates

Plate assay method

These 190 isolates were further screened for their phenol degrading capability. The isolates were streaked over minimal salt agar media with phenol as sole source of carbon. The bacterial colonies were able to grow on this media using phenol as carbon source. After the disappearance of phenol, which was indicated by color change of bromothymol blue from green to yellow [13, 2].

The colonies were picked up and spread on separate plate for getting pure culture, [Figure 1]. Five strains showing maximum growth were further screened by inoculating minimal salt agar media having phenol concentration ranging from 1mM-10 mM. From the growth observed in different concentration of phenol minimal salt media Table no.1 was tabulated. Three strains i.e.,



Control NS-1 NY-1 SP-2

Fig. 1. Screening of bacterial strains NS-1, NY-1 & SP-2 degrading phenol indicated by yellow colored colonies on minimal salt media

NY-1 (from electricity power station), NS-1 (from compost) & SP-2 (from laundry effluent) were showing the maximum tolerance to phenol at extremely stressful conditions i.e., glucose starved minimal salt media with molarities of phenol. The change in color of media from green to yellow due to acid production (decrease in pH), resulted from phenol degradation was obtained in all the four strains.

Confirmatory test

Results are shown in Table 2. NY-1, NS-1 & SP-2 had shown luxurious growth on both B[NAM] & C[Phenol+MSM] medium while NY-5 & NY-6 showed luxurious growth on B[NAM] but failed to degrade phenol and showed poor growth on C[Phenol+MSM] medium.

Characterization and optimization of bacterial isolates

On the basis of morphological and biochemical characterization, the strains NS-1 was identified as *Staphylococcus* and NY-1 & SP-2 as *Bacillus* spp. respectively [14]. The major factors incubation period, substrate concentration, pH and temperature that influence growth were optimized. Maximum degradation was obtained in the following range : for NS-1 was 72 hrs, 3-8 mM of phenol, 5.5-6.5 pH & 40-50°C temperature respectively, for SP-2 was 48-72 hrs, 4-7 mM of phenol, 5.5-7.5 pH & 20-60°C temperature and that for NY-1 was 72 hrs, 4-7 mM of phenol, 6.5-8.0pH & 40-50°C temperature [Figure 2].

Elucidation of mode of ring cleavage of phenol

Absence of yellow color on spraying catechol 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 degradation pathway were not constitutively expressed. It may be correlated that phenol degradation by ortho pathway is specified by plasmid in these species [16].

Table 1. Growth on different concentration of Phenol shown by six pure cultures

Strains	1 mM	2 Mm	3 mM	4 mM	5 mM	6 mM	7 mM	8 mM	9 mM	10 mM
NY-1	+	+	++	++	+++	++	++	+	-	-
NS-1	+	++	+++	+++	++++	++++	+++	++	+	-
SP-2	+	++	++	+++	++++	+++	++	++	-	-
NY-5	-	-	+	+	+	+	+	-	-	-
NY-6	-	-	-	+	+	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-	-

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

Table 2. Confirmatory test of phenol degrading capability on different carbon sources

Isolates	A	B	C	D
NY-1	-	++++	+++	-
NS-1	-	++++	++++	-
SP-2	-	++++	++++	-
NY-5	-	++++	+	-
NY-6		++++	+	-
Control	-	-	-	-

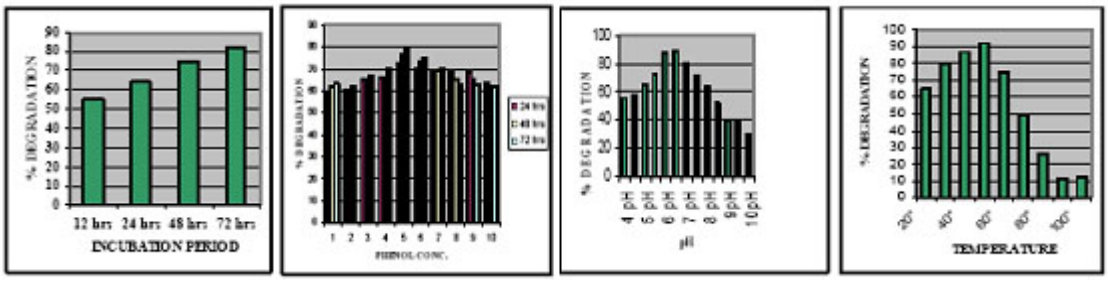


Fig. 2 (a). Effect of incubation period, substrate concentration, pH & temperature on NS-1

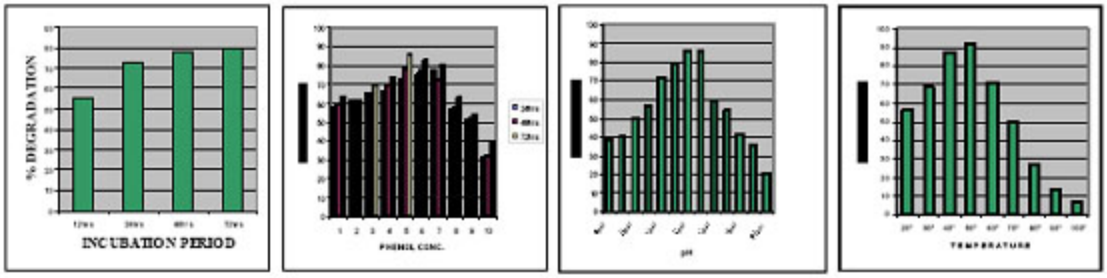


Fig. 2 (b). Effect of incubation period, substrate concentration, pH & temperature on NY-1

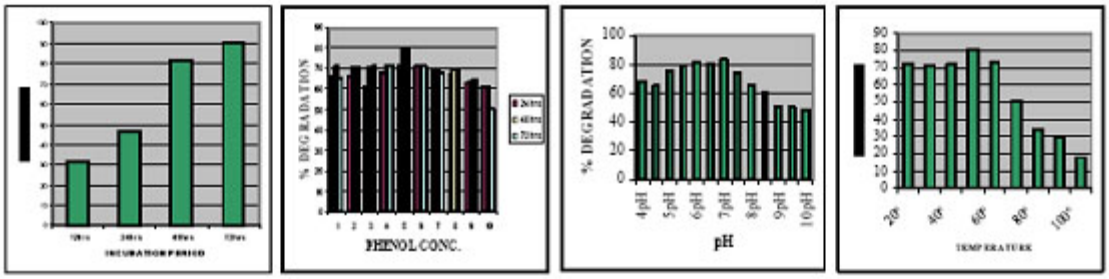


Fig. 2 (c). Effect of incubation period, substrate concentration, pH & temperature on SP-2

Antibiotic sensitivity test

Results of Antibiotic sensitivity test has been tabulated in Table No.3.

Plasmid curing

Plasmid curing was done using acridine orange taking antibiotic resistance property of the bacterial strain of interest as selectable marker. The results obtained are shown in Figure 3. The concentration of acridine orange at which the plasmid of bacterial isolates was cured was found to be in the range of 25-40 µg/ml. At 40 µg/ml, plasmid of all the three bacterial isolates got cured completely. Bacterial colonies which got cured were picked up from the original plate after comparing replica plates with original plates and inoculated in the minimal salt media. These media

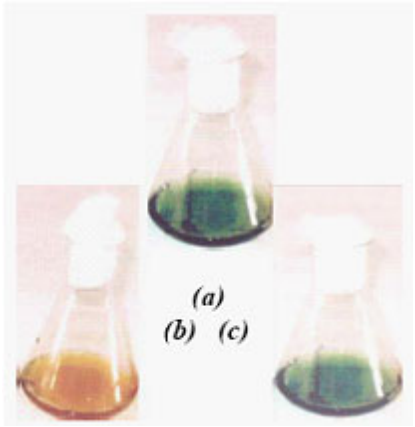


Fig.3 (a). Control (b). Phenol degraded by bacterial strains before plasmid curing (c). Phenol is not degraded by bacterial strains after plasmid curing.

Table 3. Antibiotic sensitivity test

Antibiotics	NS-1	NY-1	SP-2
	Zone of Inhibition (mm)		
Rifampicin(R-30)	12	17	14
Gentamicin (G-10)	25	21	7
Vancomycin (Va-10)	16	16	-
Amoxicillin (Am-30)	12	16	-
Ciprofloxacin (Cf-30)	24	26	40
Streptomycin (S-25)	22	26	38
Methicillin (M-30)	-	-	-
Co-Trimoxazole (Co-25)	-	-	-
AmphotericinB (Ap-100)	-	-	-
Nystatin (Ns-100)	-	-	-
Ampicillin (A-10)	20	-	-
Kanamycin (K-10)	28	20	3

were kept for incubation at 37°C for 24-72 h under shaking condition. Phenol degradation was almost negligible, which indicates that genes encoding for enzyme phenol hydroxylase was plasmid borne.

Acknowledgements

Authors are thankful to the Chairman and Managing Secretary, SBSPGI, Balawala, Dehradun, India for providing all the necessary facilities for carrying out this research work.

References

1. **Mutzel *et al.* (1996).** Isolation and characterization of a *thermophillus bacillus* strain that degrade phenol and cresol as sole carbon source at 70°C. Applied Microbiology & Biotechnology; 46: 593-596.
2. **Saadoun, I. (2004).** Recovery of *Pseudomonads* spp. From chronically fuel oil polluted soils in Jordan & the study of their capability to degrade short chain alkanes. World J. Microbiology & Biotechnology; 20:1: 43-46.
3. **Doke, J., Kudlu, P., Vijapurkar, S., Adhyapak, U. and Raman, V.K. (2005).** Application of root zone process for remediation of 2-chlorophenol. Nature Environment & Pollution Technology; 4: 327-331.
4. **Chavez, P.F., Gordillo, F. and Juez, C.A. (2006).** Adaptive responses & cellular behaviour of biphenyl degrading bacteria towards polychlorinated biphenyls. Biotechnology Advances; 24: 309-320.
5. **Zhang, Y, Lu, D, Ju, T, Wang, T, Lin, S, Zhao, Y, Wang, C, He, H and Du, Y (2013).** Biodegradation of phenol using *Bacillus cereus* WJ1 and evaluation of degradation efficiency based on a grapheme modified electrode. Int. J. Electrochem. Sci., 8, 504-519.
6. **Awan, Z U R, Shah, A H and Amja, M (2013).** Microbial degradation of phenol by locally isolated soil bacteria. Global Advanced Research Journal of Microbiology. 2(4): 72-79.
7. **Sarayu, K. and Sandhya, S. (2010).** Aerobic Biodegradation Pathway for Remazol Orange by *Pseudomonas aeruginosa*. Appl. Biochem. Biotechnol., 160: 1241-1253.
8. **Chacon, D.A., Mordles, G.O., Ordaz, N.R., Urbina, E.C., Ramirej, C.J. & Mayer, J.G. (2004).** Kinetic study of phenol hydroxylase & catechol 1, 2- dioxygenase biosynthesis by *Candida tropicalis* cell grown on different phenolic substrate. J. Microbiology & Biotechnology; 20: 695-702.

9. **Munazza, A., Nabeela, N., Rasool, S.A. and Khan, S.A. (2004).** Phenol resistant bacteria from soil: identification, characterization & general studies. Pak. J. Bot.; 36:2; 415-424.
10. **Aneja, K.R. (2003).** Experimental Microbiology, Plant Pathology & Biotechnology; 4th edition; New Age International, New Delhi: India.
11. **Van der Meer, J.R. (1997).** Evolution of novel metabolic pathways for the degradation of chloroaromatic compounds. Antonie Von Leeuwenhoek Int. J. Gen. Mol. Microbiology; 71: 159-178.
12. **Samrook, J., Fritsch, E.F. and Maniatis, T. (1989).** Molecular Cloning: A Laboratory Manual, Second edition, Cold Spring Harbor, Laboratory Press.
13. **Decker, K.M., Boerner, R.E.J. and Morris, S.J. (1999).** Scale dependent patterns of soil enzyme activity in forested landscape. Can. J. Forest Research; 29: 23-241.
14. **Bergey's Manual of Systemic Bacteriology (1984).** Edited by Buchanan, R.E. & Gibbons, N.E. (Williams & Wilkins, Baltimore).