Asian J. Exp. Sci., Vol. 22, No. 1, 2008; 55-65

Biodegradation of Phenol by Oxygenase Producing Thermophilic Microorganisms

Neetika Mathur[1]*, Vinod Kumar Nigam[2], Ashwani Kumar[1] and Purnendu Ghosh[2]

1. Department of Botany, University of Rajasthan, Jaipur - 302 004, Rajasthan, India
2. Department of Biotechnology, Birla Institute of Scientific Research, Jaipur - 302001, Rajasthan, India

Abstract : Screening and characterization of phenol degrading bacterial isolates from the soils and sewage samples of desert regions of Rajasthan was performed under batch cultivation. The degradation studies were monitored by the consumption of NADH and it was found that maximum degradation was achieved at 50°C. Out of nine phenol adapted oxygenase producing cultures, four cultures BISR 00T1, BISR 00T3, BISR 00T5 and BISR 00T8 showed maximum enzymatic activity. Various biochemical tests were also performed for their characterization.

Key words : Biodegradation, Oxygenases, Thermophilic, Phenol, Pollutants.

Introduction

Rapid industrial and economic development, human exploitation of fossil fuels and production of many synthetic compounds have introduced many compounds that are not naturally present in the environment. Many of these xenobiotics substances are toxic to the living system and their presence in the aquatic and terrestrial habitats often have serious ecological consequences. Bioremediation is emerging as most ideal technology for removing pollutants from the environment by the action of microbes or other biological systems, restoring contaminated sites and preventing further pollution (Caplan, 1993; Dua *et al*., 2002). Phenols and chlorophenols are introduced in the environment in the waste stream of several industrial operations, through its use as biocides or as by-product of other industrial operations, such as pulp bleaching with chlorine, water disinfection or even waste incineration and as degradation product of other chlorinated xenobiotics (Bollag *et al*., 1986). Other sources of phenol can be oil refineries, chemical plants, explosives manufacturing, resin manufacturing, rubber reclamation, textile mills and plants etc. Phenols have a relatively high oxygen demand (2.4 mg of O_2/mg of phenol) for degradation. These are toxic to fish at a level of above 2 mg/L and as little as 0.005mg/L of phenol can impart objectionable tastes and odour to the drinking water (Jogdand, 2003). Because of their toxic effects, phenols and chlorophenols tend to accumulate and in some cases, the contamination of soil and water is of concern (Keither and Tellard 1979; Moos *et al*., 1983; Borthwick and Schimmel 1978). Several decontamination techniques are available for the removal of contaminants from water such as

* **Corresponding Author :** Neetika Mathur, Department of Botany, University of Rajasthan, Jaipur-302004; Phone : 0141-2546381; Email : *neetikamathur@yahoo.co.in*

adsorption, ion exchange, incineration etc., but all are unable to destroy the contaminants. Biodegradation is a technique which could potentially degrade these contaminants to innocuous products (Murialdo *et al*., 2003). Microbial degradation of phenol and chlorophenols has been reported by several groups (Baker *et al*., 1980; Pignattelo *et al*., 1983; Saber and Crawford, 1985; Rozich and Colvin, 1986; Apajalahti and Salkinoja-Salonen, 1986; Radehaus and Schmidt, 1992; Ramos *et al*., 1995; Lee *et al*., 1998; Reddy and Gold, 2000; Cortes *et al*., 2002; Kirchner *et al*., 2003). Biological methods can reduce phenol down to 0.5-1 mg/L level.

Phenol hydroxylase (also known as monooxygenase) catalyses the efficient ortho-hydroxylation of phenol and ultimately its degradation (Kirchner *et al*., 2003). Phenols are first converted into more reactive dihydroxylated intermediates and then subjected to ring cleavage by molecular oxygen in presence of NAD (P) H dependent flavoprotein monooxygenases (Moonen *et al*., 2002). The ability of oxygenase to incorporate oxygen into organic compounds is important because many of the hydrophobic pollutants such as polycyclic aromatic hydrocarbons are high in carbon and hydrogen content but low in oxygen content. It is reported that enzyme catechol 2, 3-dioxygenase catalyzes the phenol degradation by meta-cleavage pathway in aerobic, sporulating, motile, rod shaped thermophilic bacteria characterized as Bacillus species with growth temperature optima of 50°-60°C (Ali Saiqa *et al*., 1998). These thermophilic bacteria can be used in treatment of phenolic waste water. The biotechnological significance and the benefits of the use of thermophilic bacteria in enhancing hydrocarbon removal under different environmental conditions were investigated. Mesophilic sources of phenol have been investigated but due to thermal instability of the enzymes the attention is being made to search for thermostable enzymes (Moiseeva *et al*., 2002).

Many of the organic compounds are first degraded to catechol or protocatechuate by oxygenases (both dioxygenases and monooxgenases). The intermediates are metabolized by a ring cleavage type of dioxgenases to either beta-ketoadipate or 2-keto-4-hydroxyvalerate and finally metabolized by TCA cycle.

In the present study, the organisms isolated from the hot desert region of Rajasthan are selected for evaluating their phenol degrading capabilities. The enzymes (whole cells) have also been characterized at various physico chemical parameters.

Materials and Methods

Isolation and growth condition

Mineral Base Medium was used for isolation of phenol degrading oxygenase producing thermophilic microorganisms. The Mineral Base Medium includes; yeast extract (2 g/L) and 5 X mineral base {NaCl 5 g/L, KH_2PO_4 1.35 g/L, K_2HPO_4 0.87 g/L, $MgSO_4.7H_2O$ 1 g/L, $CaCl_2$ 0.05 g/L, $FeCl_3$ 1.25 g/L}, 1 ml/ L of trace element solution SL6 ($CoCl_2$ 0.2 g/L, H_3BO_3 0.3 g/L, $ZnSO_4.7H_2O$ 0.1 g/L, $MnCl_2.4H_2O$ 0.03 g/L, $Na_2MoO_4.H_2O$ 0.03 g/L, $NiCl_2.6H_2O$ 0.02 g/L and $CuCl_2.2H_2O$ 0.01 g/L), and filter sterilized thiamine HCl 3 µg/l. The phenol (1 g/L) was added in the medium as a carbon source.

The pH of the medium was adjusted to 7.0 before sterilization. The phenol was added into the medium after sterilization. A number of soil and sewage samples were collected from different regions of Rajasthan. 1 g of soil/ sewage sample was added to 100 ml of Mineral Base Medium (MBM) and kept on orbital shaker at 50°C and 200 rpm for enrichment. The samples were withdrawn aseptically at different time intervals (24 h, 48 h, 72 h, etc.) and spreaded on agar plates and incubated at 50°C for 24 h. Pure colonies were obtained after several streaking. The isolates were grown in the liquid medium (100 mL) for 24 h and centrifuged at 10,000 rpm for 10 min at 4°C. The cell pellet after washing was dissolved in 2 mL of buffer and stored at 4°C till further use. Finally, the pure colonies were observed for oxygenase activity. Cell growth was measured turbidimetrically at 600 nm.

Residual phenol estimation

The consumption of phenol in culture broth was measured with respect to the standard plot of phenol by Folin's ciocalteau method (Sadasivam and Manickam, 1992).

Oxygenase assay

The oxygenase assay was performed by measuring the consumption of NADH at 340 nm using whole cells of different isolates (Kirchner *et al*. 2003). The 3 mL reaction mixture consisted of 1.9 mL of 0.1M phosphate buffer (pH 7.2), 0.5 mL substrate phenol (final concentration 116.7mM), 0.5 mL NADH (final concentration 166 µM), 0.1mL of whole cells and incubated at 50°C for 2 h. The reaction mixture was centrifuged at 10,000 rpm for 5 min and absorbance of supernatant was monitored at 340 nm against control. Enzyme activity is expressed in terms of I.U. which is defined as the amount of NADH (µM) consumed per mL of cells per minute.

Biochemical tests for the cultures

A number of biochemical tests (Carbohydrates hydrolysis, nitrate reduction, catalase, methyl red test, gelatin and starch hydrolysis etc) were performed for the initial characterization of cultures.

Results and Discussion

Nine (9) phenol adapted cultures were isolated from soil and sewage samples, which were grown in liquid medium for different time intervals and residual phenol concentration along with enzyme activity was measured. The results are given in Figure 1. Out of (9) nine cultures, seven (7) showed maximum utilization of phenol for their growth at 40 h of incubation at 50°C. In case of cultures BISR 00T1 and BISR 00T2, the utilization of phenol was minimum. By comparing the enzyme activities of 9 phenol adapted oxygenase producing isolates (Table 1) 4 cultures BISR 00T1, BISR 00T3, BISR 00T5 and BISR 00T8 were observed with maximum hydroxylation activity (oxygenase) and selected for further studies.

Growth profile

In order to achieve the maximum synthesis of oxygenase enzyme, the growth pattern of the cultures was investigated under optimal conditions (pH 7.0, temperature 50°C and rpm 200). The best isolates (BISR 00T1, BISR 00T3, BISR 00T5 and BISR 00T8) were inoculated into mineral base medium and 2 mL of samples were drawn aseptically at different time

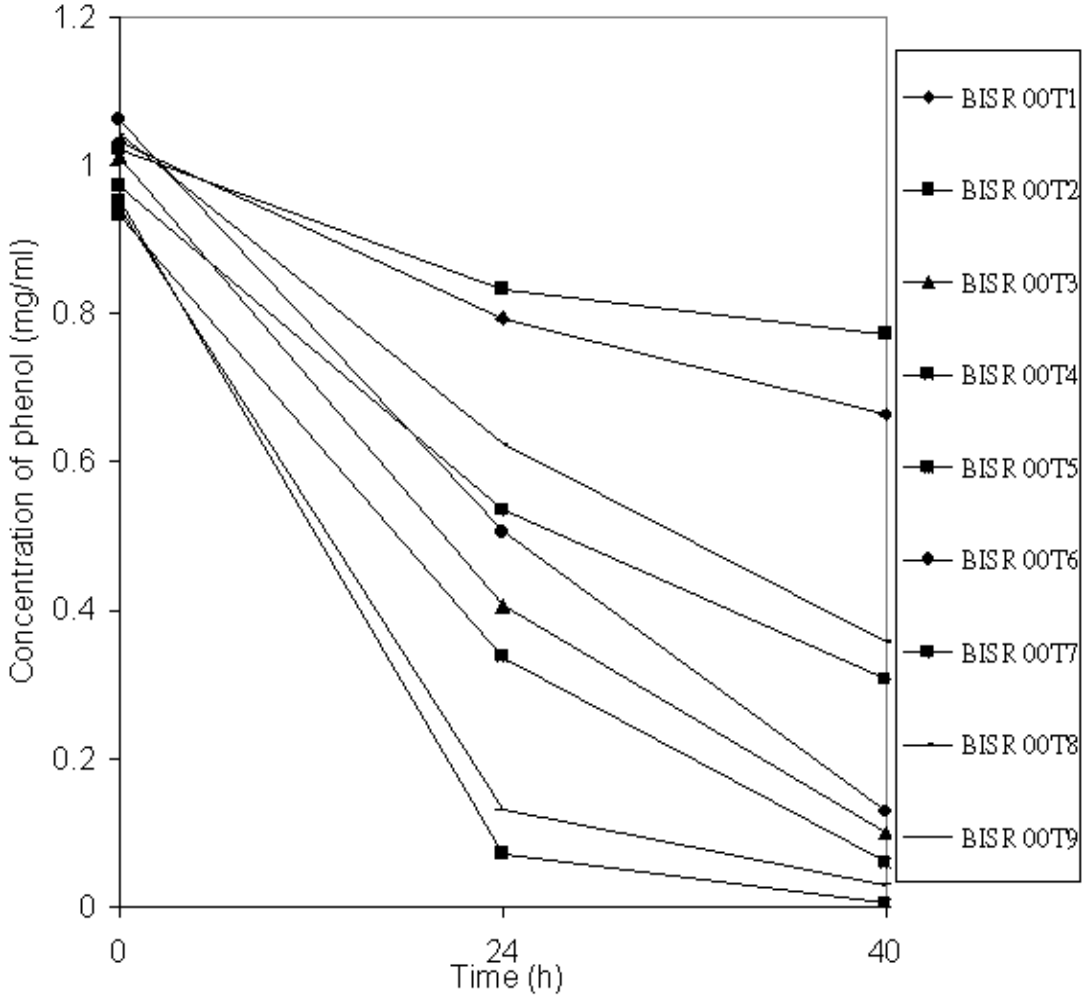

Figure 1: Measurement of residual phenol

interval for analyzing cell biomass. The supernatants were used for estimation of residual phenol (Figure 1). The results are presented in Table 2. It was observed that as the time of incubation increased, the cell biomass also increased. But this increase in cell biomass is limited upto 24 h of incubation, after that it decreased slightly and becomes almost constant.

In another experiments the cultures were inoculated in the liquid broth as mentioned above and the whole cells obtained at different time (24h, 48h and 72h respectively) were evaluated for the oxygenase activity. The results are given in Table 3. It was observed from the table that isolates grown for 48 h of incubation showed maximum enzyme activity. Later aged cells have been found with decrease in oxygenase activity. This might be because of the maturation of cells that did not favour oxygenase production.

Table 1: Evaluation of oxygenase activity by nine isolates.

Isolates	Abs at 340	NADH Residual		NADH Consumption		Enzyme
		%	Amt (µM)	%	Amt (µM)	Activity
BISR 00T1	0.1755	31.22	51.82	68.45	113.62	0.3156
BISR 00T2	0.8107	–	–	–	–	–
BISR 00T3	0.0585	10.40	17.27	89.6	148.73	0.4131
BISR 00T4	0.0748	13.3	22.09	86.7	143.92	0.3997
BISR 00T5	0.0552	9.82	16.30	90.18	149.69	0.4158
BISR 00T6	0.2410	42.87	71.17	57.13	94.83	0.2634
BISR 00T7	0.0769	13.68	22.71	86.32	143.29	0.3980
BISR 00T8	0.0763	13.57	22.53	86.43	143.47	0.3985
BISR 00T9	0.0784	13.94	23.15	86.06	142.85	0.3968
Control	0.5621	100	166	–	–	–

Table 2: Growth profile of four isolates with respect to time.

Time Period	OD at 600nm and Dry cell biomass (g/L)			
(h)	BISR 00T1	BISR 00T3	BISR 00T5	BISR 00T8
18	0.589 (0.896)	0.612 (0.93)	0.601 (0.63)	0.562 (1.05)
24	0.680 (1.05)	0.732 (1.10)	0.630 (0.70)	0.784 (1.25)
40	0.446 (1.00)	0.540 (0.95)	0.449 (0.80)	0.688 (1.05)
72	0.402 (0.80)	0.706 (0.90)	0.448 (0.65)	0.586 (0.95)

After optimizing the growth characteristics of all four isolates in the liquid broth, the enzyme oxygenase was characterized in terms of various physiological conditions such as pH, temperature, substrate concentration and substrate specificity etc.

Effect of pH on the activity of oxygenase

The effect of pH on the oxygenase activity was investigated by varying the pH of buffer in the range of 6.8-8.0 keeping all other parameters constant (temperature 50°C, phenol concentration 117 mM and incubation 1 h). The results are presented in Figure 2. The figure reveals that with increase in pH, the enzyme activity increases upto 7.6 and then decreased for culture BISR 00T1, BISR 00T5 and BISR 00T8 while in case of BISR 00T3 the oxygenase activity continuously decreased.

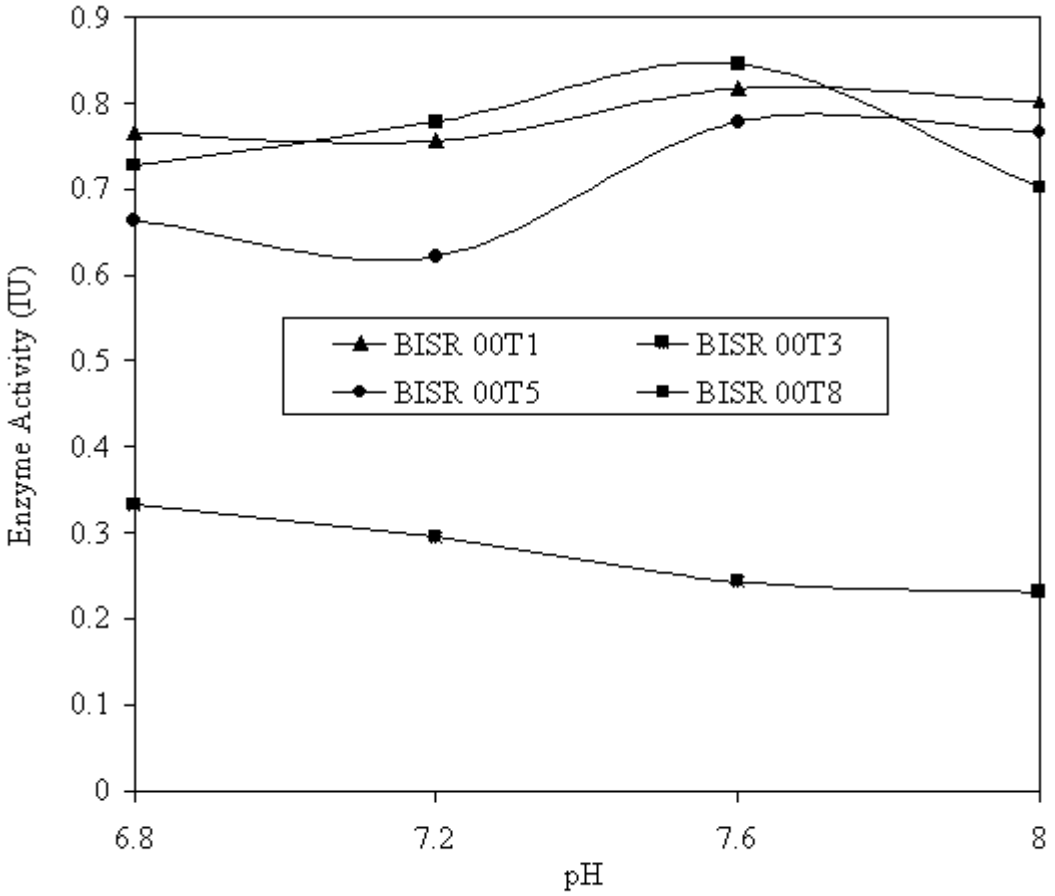

Figure 2: Effect of pH on oxygenase activity

Effect of temperature on activity of oxygenase

The effect of temperature on activity of oxygenase was performed by varying the temperatures of incubation in range of 30°C to 60°C at optimal conditions and the results are shown in Figure 3. It is clear from the figure that with the increase in temperature, the rate of phenol degradation was maximum at 50°C and then decreased for the isolates BISR 00T1, BISR 00T5 and BISR 00T8 respectively. However, for isolate BISR 00T3, the oxygenase activity was maximum at 60°C.

Effect of substrate concentration on oxygenase activity

The effect of substrate (phenol) concentration on activity of oxygenase was investigated by varying the concentration of phenol in the range of 40 mM to 240 mM keeping all other parameters constant. The result of this experiment is shown in Figure 4. The observation of figure showed that the oxygenase activity for BISR 00T1 started increasing from 40 mM to 80 mM of phenol concentration and decreased at higher concentration. For the isolate BISR 00T3, the oxygenase activity was highest at 40

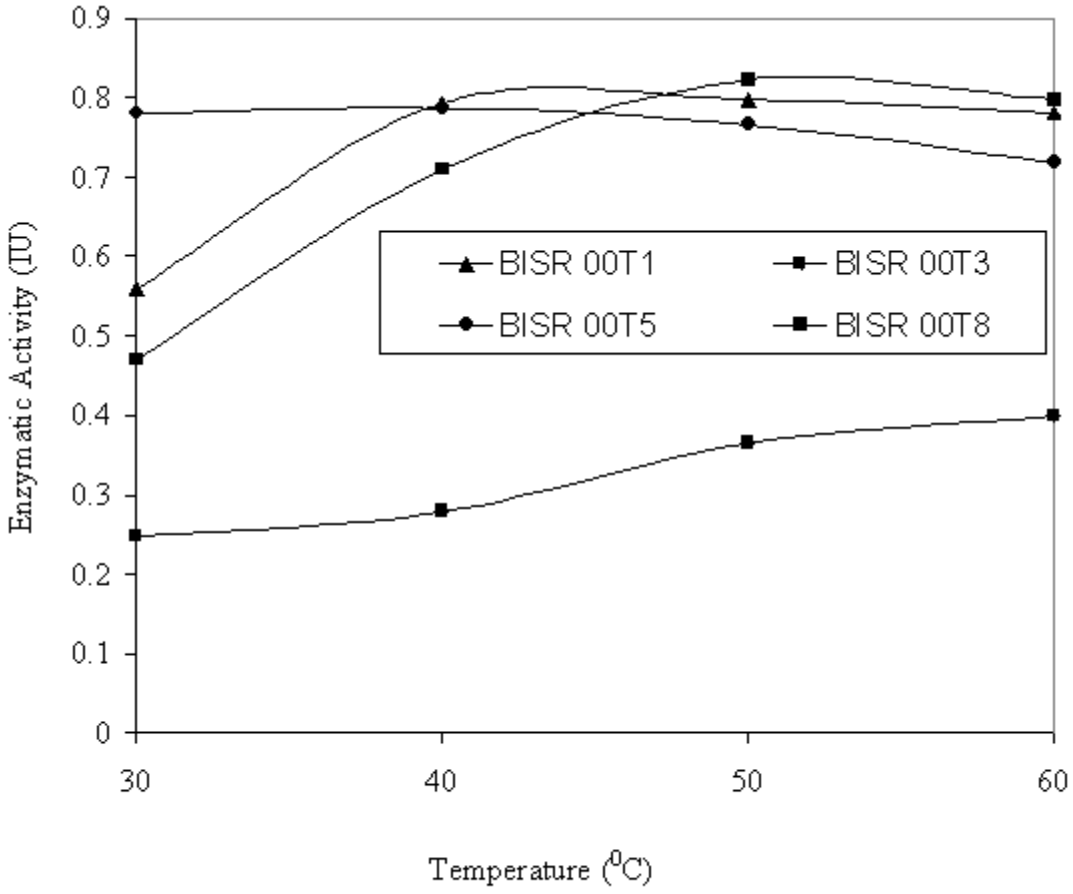

Figure 3: Effect of temperature on oxygenase activity

mM of phenol concentration. In case of BISR 00T5, the enzyme activity increased from 40 mM, then became constant at 120 mM of phenol concentration and then decreased. The utilization of phenol increased from 40 mM to 120 mM by isolate BISR 00T8, and then continuously decreased. This shows that the biodegradation of phenol varies with the different isolates. The isolate BISR 00T3 showed maximum utilization of phenol.

Effect of substrate specificity on Oxygenase activity

Different substrates (naphthalene and toluene) were used for evaluating the substrate specificity of oxygenase produced from the different thermophilic bacterial isolates. Different concentrations of naphthalene (2.13 mM to 5.3 mM) and toluene (2.25 mM to 9.0 mM) were incubated with the whole cells at optimal conditions and the oxygenase activity was monitored. The results are given in Table 4 and 5 respectively. The observation of Table 4 shows that oxygenase activity expressed by the isolates BISR 00T1 and BISR 00T5 increased from 2.13 mM to 5.3 mM of naphthalene concentration respectively.

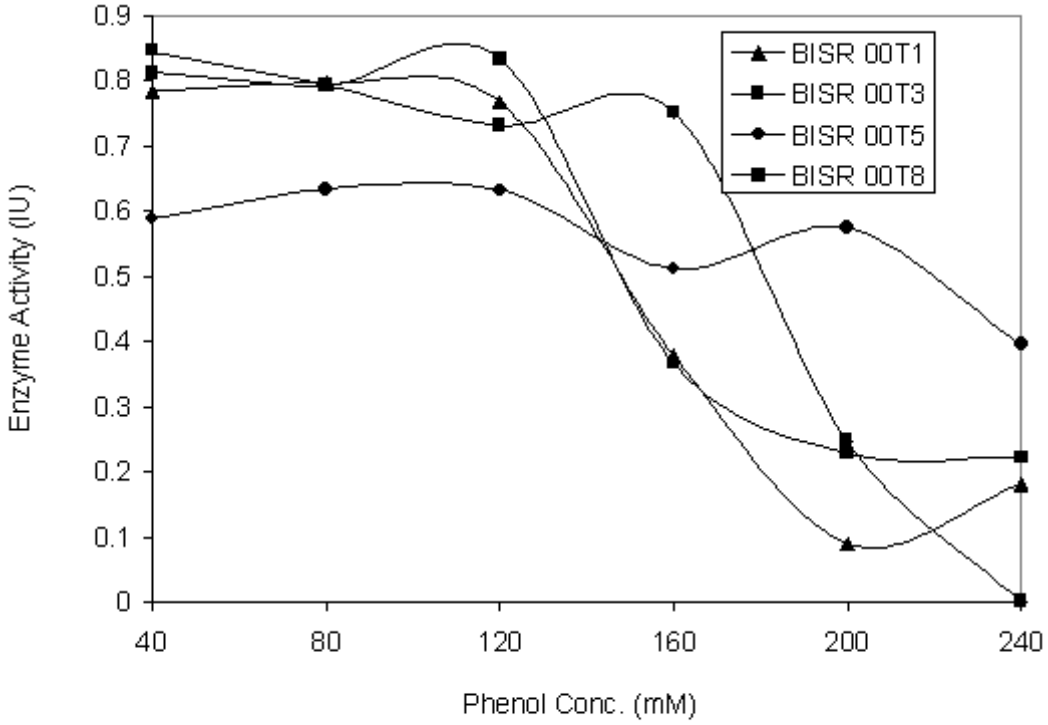

Figure 4 : Effect of phenol concentration on oxygenase activity

Table 3: Oxygenase activity of isolates at different ages of their growth.

Time Period (h)	Oxygenase activity (IU)			
	BISR 00T1	BISR 00T3	BISR 00T5	BISR 00T8
24	0.8270	0.8342	0.7956	0.4377
48	0.8440	0.8426	0.8155	0.4314
72	0.3640	0.5493	0.3135	0.7297
96	0.2468	0.5813	0.0903	0.1359

Same observation were also be noticed for isolates BISR 00T3 and BISR 00T8 with a little variation. The observation of Table 5 in case of toluene as substrate showed that the oxygenase activity for the isolates BISR 00T1 increased from 2.25 mM to 9.0 mM. For isolates BISR 00T3 and BISR 00T8 the oxygenase activity increased from 2.25 mM to 4.5 mM and then decreased. The oxygenase activity was highest at 2.25 mM of toluene concentration in case of the isolate BISR 00T5 and then decreased.

Table 4: Effect of naphthalene concentration on oxygenase activity.

Naphthalene Concentration (mM)	Oxygenase Activity (IU)			
	BISR 00T1	BISR 00T3	BISR 00T5	BISR 00T8
2.13	0.8965	0.8857	0.8112	0.8702
3.2	0.9120	0.8890	0.7382	0.8746
5.3	0.9148	0.8346	0.8651	0.8667

Table 5: Effect of toluene concentration on oxygenase activity.

Naphthalene Concentration (mM)	Oxygenase Activity (IU)			
	BISR 00T1	BISR 00T3	BISR 00T5	BISR 00T8
2.25	0.8997	0.00	0.8445	0.7935
4.5	0.9131	0.9089	0.8380	0.8011
9.0	0.9159	0.8754	0.8077	0.6033

Table 6: Results of Biochemical Tests.

Phenol Adapted Oxygenase Producing Cultures	Carbohydrates Test			Nitrate	Catalase	Methyl Red	Gelatin	Starch
	Dextrose	Sucrose	Lactose					
BISR 00T1	+	+	-	+	+	+	+	-
BISR 00T2	+	+	-	+	+	-	-	-
BISR 00T3	+	+	-	+	+	-	-	-
BISR 00T4	+	+	-	+	+	+	+	-
BISR 00T5	+	-	-	+	+	-	-	-
BISR 00T6	+	+	-	+	+	+	+	-
BISR 00T7	+	+	-	+	+	+	+	-
BISR 00T8	+	+	-	+	+	+	+	-
BISR 00T9	+	+	-	+	+	+	+	-

Biochemical Test

All the cultures have been characterized by different biochemical tests and the results are shown in Table 6. The table revealed that all isolates are able to degrade carbohydrates (glucose and sucrose) except lactose. All the isolates showed positive results for catalase and nitrate reduction test but negative for starch hydrolysis.

Conclusion

Out of nine phenol adapted oxygenase producing isolates from soil and sewage samples, four isolates showed maximum oxygenase activity at 50°C. The isolate BISR 00T3 showed maximum utilization of phenol during the enrichment. The growth profile showed that the enzyme production was maximum at 48 h of incubation. For isolates BISR 00T1, BISR 00T5 and BISR 00T8, the optimum pH was 7.6 and optimum temperature was 50°C for oxygenase activity while for the isolate BISR 00T3, the optimum pH was 6.8 and optimum temperature was 60°C respectively.

References

Alis S, Lafuente R.F. and Cowan D.A. (1998): Meta-pathway degradation of phenolics by thermophilic Bacilli, *Enzyme and Microbial Technology*, **23**, 462-468.

Apajalahti Jha and Salkinoja- Salonen M.S. (1986): Degradation of polychlorinated phenols by Rhodococcus chloro-phenolics. *Applied Microbial Biotechnology*, **25**, 62-67.

Baker M.D., Mayfield C.I. and Inniss W.E. (1980): Degradation of chlorophenols in soil, sediments and water at low temperature. *Water Res*., **14**, 1765-1771.

Bollag J.M., Helling C.S. and Alexander M.J. (1986): 2,4-D metabolism. Enzymatic hydroxylation of chlorinated phenols. *J. Agric. Food Chem*., **16**, 826-828.

Borthwich P.W. and Schimmel S.C. (1978) : Toxicity of pentachlorophenol and related compounds to early life stages of selected estuarine animals. In: K Ranga Rao (ed.) Pentachlorophenols: Chemistry, Pharmacology and Environmental Technology, Plenum Press, New York.

Caplan J. A. (1993): The worldwide bioremediation industry- prospects for profit, Tibtech. 11, pg. 320-323.

Cortes D., Garrios-Gonzalez J. and Tomasini A. (2002): Pentachlorophenol Tolerance and removal by Rhizopus nigricans in solid-state culture. *Proc. Biochem*., **37**, 881-884.

Dua M., Sethunathan N. and Johri A.K. (2002): Biotechnology and bioremediation: success and limitations. *Appl. Microbio. Biotechnol*. **59**, 143-152.

Jogdand S.N. (2003): Biotechnology for hazardous waste management- Environmental Biotechnology, 139-140.

Keither L.H. and Tellard W.A. (1979): Priority pollutants. A Perspective view. *Environ. Sci. Technol*., **13**, 416-423.

Kirchner U., Westphal A.H., Muller R. and Berkel W.J.H. (2003): Phenol Hydroxylase from Bacillus thermoglucosidasius A7, a Two-protein Component Monooxygenase with a Dual Role for FAD, *The Journal of Biological Chemistry*, **278**, 47545-47553.

Lee S.G., Yoon B.D., Park Y.H., Oh H.M. (1998): Isolation of novel pentachlorophenol-degrading bacterium, Pseudomonas sp. Bu. 34. *J. of Appl. Microbiol*., **26**, 85-90.

Moiseeva O.V., Solyanikova I.P., Kaschabek S.R., Groning J., Thicl M., Golovlena L.A. and Schlomann M. (2002): *J. Bacteriology*, **184**, 5282-5292.

Moonen M.J.H., Fraaije M.W., Rietjens I.M.C.M., Laane C. and van Berkel W.J.H. (2002): *Adv. Synth. Catal*., **344**, 1023-1035.

Moos L.P., kirsch E.J., Wukasch R.F. and Grady

C.P.L. (1983): *Pentachlorophenol Biodegradation. Water Res*., **17**, 1575-1584.

Murialdo S.E., Fenoglio R., Haure P.M. and Gonzalez J.F. (2003): Degradation of phenols and chlorophenols by mixed and pure cultures. *Water S. A*., **29**, 457-463.

Pignatello J.J., Martison M.M., Steiert J.G., Carlson R.E. and Crawford R.L. (1983): Biodegradation and photolysis of pentachlorophenol in artificial freashwater streams. *Appl. And Environ. Microbio*., **46**, 1024-1031.

Radehaus P.M. and Schmidt S.K. (1992): Characterization of novel Pseudomonas sp. that mineralizes high concentrations of pentachlorophenol. *Appl. Environ. Microbiol*., **58**, 2879-2885.

Ramos J.L., Duque E., Huertas M.J. and Haisour A (1995): Isolation and expansion and expansion of the catalic potential of a Pseudomonas putida strain able to grow in the presence of high concentrations of aromatic hydrocarbons. *J. Bacteriol*., **177**, 3911-3916.

Reddy G.V.B. and Gold M.H. (2000): Degradation of pentachlorophenol by Phanerochaete chrysosporium: Intermediates and reactions involved. *Microbiol*., **146**, 405-413.

Rozich A.F. and Colvin R.J. (1986): Effects of glucose on phenol biodegradation by heterogenous populations. *Biotechnol and Bioeng*., **28**, 956-971.

Saber D.L. and Crawford R.L. (1985): Isolation and characterization of Flavobacterium strains that degrade pentachlorophenol. *Appl. and Environ. Microbiol*., **50**, 1512-1518

Sadasivam S. and Manickam A. (1992): Biochemical Methods for Agricultural Sciences, . Wiley Eastern Limited publication. 187.