

2

## Biodegradation of phenol using bacteria from different brackishwater habitats

Imelda Joseph\* & V Chandrika

Central Marine Fisheries Research Institute, Post Box 1603, Tatapuram P O, Cochin 682 014, Kerala, India

Received 6 July 1998, revised Aug 16 1999

Bacterial degradation of phenol (100 to 800 ppm) was studied. It was observed that bacterial diversity and the rate of phenol biodegradation were influenced by the phenol concentrations in the medium. The rate of biodegradation was reduced at higher concentrations of phenol<sup>1</sup> and *Pseudomonas* spp and *Vibrio* spp were mainly involved in the process. The distribution of bacterial genera in the medium was dependent on the compound and its concentration. *Pseudomonas* and *Vibrio* were present in all concentrations of phenol, o-cresol and orcinol. Whereas, *Bacillus* and *Alcaligenes* could tolerate only up to 200 ppm of these compounds in the medium. *Flavobacterium* was absent in o-cresol, while it could tolerate 500 ppm of orcinol and 200 ppm of phenol. *Cytophaga* tolerated 300 ppm of orcinol, and 100 ppm of phenol and o-cresol. From the present study, it is concluded that in case of an accidental spill of these phenolic compounds many naturally present bacteria could be of great help in their biodegradation and purification of the system thereafter.

Biodegradation is a difficult process to be quantified since living organisms in a dynamic environment carries it out. The occurrence and degradation of phenolic compounds by bacteria, the mechanisms involved in the process, the catabolic pathways and intermediate products of degradation and optimal conditions required for the processes are studied by many workers<sup>1-4</sup>. Microorganisms that can oxidize phenols were also reported<sup>4,5</sup>. Due to high volatility, phenols impart bad taste and odour to the aquatic environment even at part/billion levels in some cases. A wide range of phenol concentration (0.08 to 1800 ppm) pose serious pollution problem, adversely affecting the organisms of food chain and fish population by interfering with carbohydrate, protein and lipid metabolism, ions transport, nerve conduction and energy production and at molecular levels due to oxidative phosphorylation in fishes<sup>6,7</sup>. Desired level of phenol for the protection of public health is 3.5 ppm and the level of controlling undesirable taste and odour of ambient water<sup>8</sup> is 0.3 ppm.

Microorganisms possess a remarkable adaptive capacity and can develop resistance to many toxic compounds. But all of them are not able to degrade toxic compounds like phenol. Only some enzymes called constitutive enzymes are present in an organism in physiologically significant amount under all conditions. Others are formed as and when needed only<sup>9</sup>. It is therefore necessary to adapt bacteria to

the compound introduced in the environment to facilitate the induction of the enzymes. Predominance of such bacteria in a mixed culture would effectively remove the pollutant from the environment<sup>10</sup>.

The objective of this investigation was to determine the ability of specifically adapted bacteria to degrade phenol, when used as sole source of carbon in mineral salts medium at higher concentrations than that are generally present in the natural environment.

### Materials and Methods

Bacteria were isolated from a coconut husk retting ground, a mangrove swamp, a free flowing backwater system and seasonal and perennial aquaculture ponds. During the process of coconut husk retting, polyphenols are reported to be leached out into the ambient water, thus causing heavy organic pollution<sup>11</sup>. Many workers also report the presence of phenolic compounds in mangrove swamps<sup>12,13</sup>. Apart from natural phenolic compounds leached from plant degradation, synthetic compounds also occur in aquatic ecosystems from effluents of oil refineries and other factories where phenolic resins are manufactured<sup>14</sup>.

Method used in selecting or adapting bacteria to degrade phenol was by primary enrichment on mechanical shaker<sup>2</sup> and further screening and quantitative study by static flask culture method<sup>15</sup>. One part of culture enriched by mechanical shaking were inoculated in 250 ml conical flasks containing 50 ml of fresh sterilized mineral salts medium

\*Present address: NRC of CIBA, Narakkal, Cochin-682 505, Kerala, India

5(MSM) containing 0.25 µg of vitamin B<sub>12</sub> (Table 1). Phenol (100 to 800 ppm), o-cresol (100 to 500 ppm) or orcinol (100 to 500 ppm) served as the sole source of carbon in the medium. The pH of the medium was adjusted at 7.0 to 7.2. The culture flasks were incubated at room temperature (28±2°C). At weekly intervals, sub culturing was done (Sub culture I, Sub culture II and Sub culture III). Triplicates of each concentration were maintained.

Bacterial cells were separated by centrifugation and the supernatant was filtered and used to estimate residual phenol calorimetrically following the 4-amino antipyrine method<sup>16</sup>. The mixed cultures were pour plated on mineral salts agar (MSM + 1.5% agar) and seawater agar. Seawater agar was used for maintaining the culture on slants. The colonies were isolated and characterized using standard morphological, physiological and biochemical tests<sup>17,18</sup>. The presence of *Pseudomonas* spp was confirmed with the help of cytochrome oxidase test. The scheme used for identification was that of Simidu & Aiso<sup>19</sup>.

Two way ANOVA was done for analyzing statistical significance of biodegradability of phenol over three sub cultures and over concentrations<sup>20</sup>.

## Results

Enrichment experiments and biodegradability studies have demonstrated that phenol could be significantly biodegraded. The rate of biodegradation ranged from 46 to 100% for the bacterial samples collected from four different locations (Table 2). When phenol was added up to 300 ppm, complete degradation was observed within three days of incubation. As expected, the rates of degradation showed significant increase with the sub culture and were highest for sub culture III (Table 3).

Phenol utilizing bacteria were present at all the four stations sampled. They were mostly Gram negative, aerobic, and small to medium sized rods. Both motile and non-motile forms were present. Based on morphological and biochemical characteristics, the colonies were tentatively identified as *Alcaligenes*, *Cytophaga*, *Enterobacteriaceae*, *Flavobacterium*, *Pseudomonas*, and *Vibrio*. Gram positive *Bacillus* sp. and *Micrococcus* sp. were also present in the samples.

The percentage distribution of bacteria at different concentrations of phenol (100 to 800 ppm), o-cresol (100 to 500 ppm) are given in Table 4. At higher

Table 1—Composition of mineral salts medium (MSM)

K <sub>2</sub> HPO <sub>4</sub>	—	1.0 g
MgSO <sub>4</sub>	—	0.2 g
NaCl	—	15.0 g
CaCl <sub>2</sub>	—	0.1 g
FeCl <sub>2</sub>	—	0.02 g
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	—	1.0 g
Distilled water	—	1.0 liter
Phenol	—	A>
pH	—	7.0 to 7.2

Table 2—Biodegradability of phenol using mixed culture of bacteria. [Average of triplicate (9 days)]

Phenol conc (ppm)	Biodegradation (%)		
	I SC	II SC	III SC
100	a) 100	100	100
	b) 100	100	100
	c) 100	100	100
	d) 100	100	100
200	a) 100	100	100
	b) 100	100	100
	c) 100	100	100
	d) 100	100	100
300	a) 100	100	100
	b) 100	100	100
	c) 100	100	100
	d) 85	95	100
400	a) 90	100	100
	b) 80	92	100
	c) 75	90	100
	d) 72	83	98
500	a) 80	95	100
	b) 80	92	100
	c) 70	80	90
	d) 68	76	80
600	a) 78	80	85
	b) 75	77	79
	c) 68	74	78
	d) 65	70	73
700	a) 74	78	80
	b) 70	73	78
	c) 65	69	76
	d) 63	66	68
800	a) 60	66	48
	b) 48	54	60
	c) 48	52	54
	d) 46	50	52

a) coconut husk retting area; b) mangrove swamp; c) backwater and d) aquaculture ponds

concentrations of phenol (500 ppm and above), only *Pseudomonas* and *Vibrio* were present. Most of the genera, which utilized phenol, were also present in o-cresol and orcinol. Two way ANOVA indicates that F

Table 3—Two way ANOVA for biodegradability of phenol over sub cultures and over concentrations

Source	Degrees of Freedom	Sum of squares	Mean sum of squares	F	Remarks
(A) Coconut husk retting area					
Sub culture	2	214.32	107.16	4.72	sig (5 %)
Concentration	9	6410.30	712.26	31.57	sig (5 %)
Error	18	408.68	22.70		
Total	29	7033.30			
(B) Mangrove swamp					
Sub culture	2	232.20	116.11	6.58	sig (1 %)
Concentration	9	7742.70	860.30	48.75	sig (1 %)
Error	18	37.6	17.65		
Total	29	8292.50			
(C) Backwater system					
Sub culture	2	321.58	160.79	7.68	sig (1 %)
Concentration	9	8006.31	889.59	42.50	sig (1 %)
Error	18	376.81	20.93		
Total	29	8704.69			
(D) Aquaculture ponds					
Sub culture	2	301.24	150.62	7.99	sig (1 %)
Concentration	9	7546.60	833.51	44.46	sig (1 %)
Error	18	339.50	18.87		
Total	29	8187.31			

values for all the four stations between sub cultures and concentrations were significant at  $P < 0.01$  and  $P < 0.05$  respectively (Table 3).

### Discussion

Enrichment culture was done by shake flask culture technique for faster multiplication of phenol using bacteria and for better adaptation to phenol. For further studies, the static-culture flask biodegradation method was followed in the present investigation using mixed bacterial isolates from different locations. Bunch & Chambers<sup>21</sup> and Tabak *et al.*<sup>15</sup> followed a similar approach.

The source of inoculum showed considerable influence on the biodegradation of phenol in the present study. Bacteria from the coconut husk retting area, with high load of organic wastes, showed maximum rate of biodegradation. Adaptation of bacteria to phenol had significant correlation with biodegradation. It was reported earlier that bacteria should be well adapted to metabolize phenol, hydroxyphenols *etc.*, when cultured in MSM<sup>2</sup>. In the present study also, biodegradation studies were conducted after adapting the bacteria from different locations by primary enrichment technique. Also on

determining biodegradation rates following static culture flask, it was found that many bacteria could tolerate phenol concentration as high as 800 ppm after proper adaptation. A similar observation was reported earlier in which the most adapted group as sub culture III in the present study utilized highest quantity of phenol<sup>15</sup>.

It has been shown that 1000 ppm to 2400 ppm of phenol can be degraded by bacteria or fungi after proper adaptation<sup>1,3,11,12</sup>. In the present study, mixed bacterial populations degraded 100 ppm to 800 ppm of phenol. Phenol pollution is not a serious problem at any of the locations sampled, however, the study is important if the aquatic environments get polluted by. Of different types of pollutant treatments are followed, biodegradation is preferred since it is economical and harmless<sup>4</sup>.

The results indicate that phenol adapted bacteria could utilize up to 300 ppm of phenol in the medium within 9 days. It is also observed that the rate of biodegradation is dependent on the concentration of phenol in the medium (Table 2). It was interesting to note that maximum degradation of phenol in the initial two to three days and only marginal increase in the proceeding days. It was reported that rate of

Table 4—Percentage distribution of bacteria at different concentrations of phenol, o-Cresol and orcinol in biodegradation experiments

	Phenol conc (ppm)							
	100	200	300	400	500	600	700	800
<i>Alcaligenes</i>	18.8	12.5	14.3	12.5	16.6	—	—	—
<i>Bacillus</i>	9.1	—	—	—	—	—	—	—
<i>Cytophaga</i>	8.7	—	—	—	—	—	—	—
<i>Enterobacteriaceae</i>	9.1	12.5	14.8	12.5	16.7	—	—	—
<i>Flavobacterium</i>	8.9	12.5	—	—	—	—	—	—
<i>Micrococcus</i>	9.1	12.5	—	28.6	12.5	16.7	—	—
<i>Pseudomonas</i>	27.3	25	28	37.5	33.3	33.4	50	66.3
<i>Vibrio</i>	9	25	14.3	25	16.7	66.6	50	33.7
	o-Cresol conc (ppm)							
	100	200	300	400	500			
<i>Alcaligenes</i>	15.3	—	—	—	—			
<i>Bacillus</i>	7.6	—	—	—	—			
<i>Cytophaga</i>	7.9	—	—	—	—			
<i>Pseudomonas</i>	46.1	77.4	66.7	80	82			
<i>Vibrio</i>	23.1	22.6	33.3	20	18			
	Orcinol conc (ppm)							
	100	200	300	400	500			
<i>Alcaligenes</i>	10.0	—	—	—	—			
<i>Bacillus</i>	30.0	14.5	—	—	—			
<i>Cytophaga</i>	10.0	6.6	4.3	—	—			
<i>Flavobacterium</i>	10.0	13.0	12.3	6.7	6.7			
<i>Pseudomonas</i>	20.0	42.6	40.6	40.0	37.3			
<i>Vibrio</i>	20.0	23.3	42.8	53.3	56.0			

biodegradation to be more during the first phase of incubation period<sup>2</sup>.

Phenololytic microorganisms belong to bacteria, fungi and actinomycetes. Among bacteria, those belonging to genera, *Azotobacter*, *Alcaligenes*, *Pseudomonas*, *Flavobacterium*, *Clostridium*, *Micrococcus*, *Vibrio*, *Sarcina* etc. were reported to utilize phenolics as sole source of carbon<sup>5,22</sup>. In the present study, bacterial genera, which could tolerate phenol at different concentrations, were *Alcaligenes*, *Bacillus*, *Cytophaga*, *Enterobacteriaceae*, *Flavobacterium*, *Micrococcus*, *Pseudomonas* and *Vibrio*. Same genera were encountered in orcinol and o-cresol also. At concentrations above 400 ppm only *Pseudomonas* sp. and *Vibrio* sp. were present.

The chemical structure and concentration of the substrates are important considerations in biodegradation process<sup>23</sup>. Though chemical and some biological processes bring about some changes in the chemical structure of the compound introduced (phenol) thereby reducing the risk of environmental

pollution. Thus it forms an effective method of pollutant treatment in environment when other processes are not economically feasible and socially acceptable.

### Acknowledgement

The first author is thankful to the Indian Council of Agricultural Research (ICAR), New Delhi, for the financial assistance provided during the period of this study.

### References

- 1 Gray P H H & Thornton H G, *Zentral Bacteriol Parasitenk*, Abstract II (1928) 73.
- 2 Tabak H H, Chambers C W & Kabler P W, *J Bacteriol*, 87 (1964) 910.
- 3 Iturriaga R & Rheinheimer G, *Keiler Meeresforschung*, 28 (1972) 1.
- 4 Shivaraman N & Parhad N M, *Indian J Microbiol*, 25 (1985) 79.
- 5 Aaronson S, in *Experimental microbial ecology* (Academic Press, New York & London) 1970, 65.
- 6 Desiah, D, *Pentachlorophenol*, 12 (1978) 277.

- 7 Gupta, S, in *Proceedings of symposium on assessment of environmental pollution* (The Academy of Environmental Biology, Muzaffarnagar India) 1985, 151.
- 8 Anon, *Ambient water quality criteria for phenol*, EPA 440/5-0-066 (United States Environmental Protection Agency), 1980.
- 9 Slater J H & Somerville H J, in *Microbial technology*, edited by A T Bull, D C Ellwood & R Ratledge (Cambridge University Press, Cambridge) 1979, 128.
- 10 Sastry C A, *Encology*, 1 (1986) 29.
- 11 Joseph, I, in *Biodegradation of phenolic compounds in different ecosystems in Cochin*, Ph. D, thesis, Cochin University of Science and Technology, India, 1997.
- 12 Karanth N G, Lokbharathy P A & Nair S, *Indian J Mar Sci*, 4 (1975) 215.
- 13 Gomes H P & Mavinkurve S, *Mahasagar—Bull Natl Inst Oceanogr*, 15 (1982) 111.
- 14 Jorgenson S E, *Vatten*, 27 (1971) 434.
- 15 Tabak H H, Quave A S, Mashmi I C & Barth F E, *J Water Pollut Control Fed*, 53 (1981) 1503.
- 16 APHA, in *Standard methods for examination of water and waste water*, (ed 14) (American Public Health Association Inc, New York) 1975, 574.
- 17 Gaby W L & Hadley C, *J Bacteriol*, 74 (1957) 356.
- 18 Gaby W L & Free E, *J Bacteriol*, 76 (1958) 442.
- 19 Simidu U & Aiso K K, *Bull Jap Soc Sci Fish*, 28 (1962) 1135.
- 20 Snedecor G W & Cochran W G, in *Statistical methods*, (Oxford & IBH Publ Co, New Delhi) 1967, 593.
- 21 Bunch R L & Chambers C W, *J Water Pollut Control Fed*, 39 (1967) 181.
- 22 Visser S A, Lamontagne G, Zoulalian V & Tassier A, *Environ Contam Toxicol*, 6 (1977) 455.
- 23 Bourquin A W, in *Genetic control of environmental pollutants*, edited by O S Gulbert & H Alexander, (Plenum Press, New York), 1984, 97.

D. (Mrs.)  
Scientist

H, Ph.D.

Central Marine Fisheries Research Institute  
P. Box No. 1803  
Cochin - 682 014, India,