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RESEARCH ARTICLE

Biodegradation pattern of polyphenols released during husk retting in different bacterial isolates *in vitro*

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SUMMARY : Phenol and its derivatives are known as major organic pollutants. Despite their often unusual structure only few of these compounds appear to cause environmental problems due to incomplete degradation. Certain efficient microorganisms make use of these organic pollutants for their growth and functioning of cellular process by electron transport mechanisms and help in transformation of otherwise stable end products. Since microbes have the potential to degrade phenolic compounds, and considering the toxicity of phenols, several studies have been focused in this aspect. The present study is aimed to analyze the competency of degrading capacities in ten microbial strains in different time intervals and to determine the most efficient strain capable of surviving in harsh or polluted environment.

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Phenol exists in the environment due to both natural and anthropogenic causes *viz.*, coir retting(where the coconut husk is steeped into water for liberating the fibres from husk), coal refining, petroleum refining, phenol manufacture, pharmaceuticals etc. Phenols consist of monohydric phenol, catechol, cresols, chlorophenols, hydroquinol, resorcinol, xylenols and aminophenols. Phenol concentrations up to 17,500 mg/l in industrial wastewaters have been reported (Carbajo *et al.*, 2010). Phenol removal via biological method is the most favoured process because of the possibility of complete mineralization of phenol (El Naas, 2009). Aromatic compounds, being rich in carbon content, once the rings are cleaved by the organisms, the products (organic acids) formed, enter into the energy cycle. Normally when microbial strains are exposed to highly polluted or toxic environment many of them perish while certain highly capable and efficient "newer" strains when exposed to an limiting environment develop novel strategies in their cellular pathways wherein they are enriched slowly and steadily in the process of "acclimation" to cope up with the polluted environment by acquiring new genetic properties to live in the alien environment by mutation, substitution and expression of the new gene.

Micro-organisms that degrade phenol were isolated as early as in 1980 (Evans, 1948) and biodetoxification of phenol was proven as a viable method for remediating phenol containing wastewater. Many organisms can degrade phenol at low concentrations including *Alcaligenes eutrophus*, *Bacillus stearothermophilus*, *Pseudomonas* , *Streptomyces* , *Trichospora* and *Rhodotorula rubra* (Pradhan and Ingle, 2003). Biosorption technique was more advantageous than the other methods in phenol removal (35 to 100 %) followed by immobilization technique.

The studies revealed that the cultures utilized the substrate as sole carbon source. The growth response to the utilization of phenols showed a corresponding increase reported an inverse relation between diversity of bacteria and phenol concentration. Many related studies have

shown inverse relationship between growth and phenol concentration. Phenol catabolism was confirmed through the detection of the intermediary products namely catechol (at the earlier stage) and cis, cis-muconic acid (at the later stage). The greater the number of substituent's in the structure of phenol, the more toxic, recalcitrant and less degradable it becomes.

In the first step of the aerobic pathway for the biodegradation of phenol, molecular oxygen is used by the enzyme phenol hydroxylase to add a second hydroxyl group in ortho-position to the one already present in which reaction requires a reduced pyridine nucleotide (NADH2). The resulting catechol (1, 2-.dihydroxybenzene) molecule can then be degraded via two alternative pathways. Depending on the type of strain, the catechol then undergoes a ring cleavage that can occur either at the ortho position thus initiating the ortho pathway that leads to the formation of succinyl Co-A and acetyl Co-A or at the meta position thus initiating the meta pathway that leads to the formation of pyruvate and acetaldehyde (Agarry, 2008). The organisms which utilize phenol by aerobic pathway are *Acientobacter calcoceticus*, *Pseudomonas* species and *Candida tropicalis* and most of the eukaryotes typically employ ortho pathway. The aerobic genus *Pseudomonas* has been subject to various studies and its versatility to utilize a wide spread of aromatic substrates makes it an attractive organism for use in waste water treatment applications (Basha *et al.*, 2010).

Micro-organisms that degrade cellulose are abundant in nature. They include both aerobic and anaerobic fungi and bacteria, many of which grow under extreme conditions of temperature and pH. In general, methyl-substituted aromatic compounds are degraded via the meta cleavage pathway, whereas xenobotics like chloroaromtic compounds are mineralized via the ortho cleavage pathway (Knackmuss, 1984).

One adaptive mechanism enabling several *Pseudomonas* strains to grow in the presence of membrane-disrupting compounds is the isomerization of cis-unsaturated fatty acids to trans-unsaturated fatty acids. Thus, greater is the Cis/Trans ratio higher is the indication of environmental stress. Joseph and Chandrika (1999) studied biodegradation of phenol (100 to 800 ppm) using several bacterial cultures. *Pseudomonas* and *Vibrio* were reported to be present in all concentrations of phenol, while *Bacillus* and *Alcaligenes* could tolerate only up to 200 ppm of these compounds in the medium by Joseph and Chandrika (1999).

EXPERIMENTAL METHODOLOGY

Kadinamkulam lake (Lat 8º 35' to 8º 45' N and Lon. 76º 45' to 76º 56' E) in the Thiruvananthapuram district, one of the major retting zones dotting the coastal belt of Kerala, India was selected for this study. Five sites were selected for the present study which included Kadinamkulam (KK), Kandavila (KV), Madanvila (MV), Kottaramthuruthi (KT) and Puthukurichi (PK). Polyphenols from the husks are constantly leached out into the surrounding steep liquors during the course of retting and the relatively high percentage of such polyphenols in coconut husks are the very reason for the delay in the completion of the retting process.

The methods followed for growth of cultures in phenol, biodegradation studies *in vitro*, are presented here. Polyphenol concentration ranged from 0.0003 to 1.897 mgL^{-1}. Polyphenol concentration was high during the initial period of retting and the concentration decreased as retting proceeded to middle stage of retting (Reshma, 2008).

Growth rate experiments of the isolates were carried out for a period of four days, in minimal media. The first sample (time zero) was taken immediately after substrate addition for each isolate and growth was recorded in terms of optical density (OD) at A 600. The samples were incubated at room temperature and 1ml aliquots of the isolates were taken each day at 24 h interval for a period of four days, in order to monitor the OD600. Two sets of control were maintained. One set of broth added with respective cultures without phenol and another set of control with phenol without culture. The experiment was done in triplicate.

From the samples prepared for growth rate experiments, 1ml from each sample was aliquoted every 24, 48, 72 and 96 h, in order to monitor the residual phenol in the sample. The residual phenol in the sample was determined following Bray and Thorpe (1954). The amount of residual phenol in the sample was measured using a standard graph prepared from catechol. The bacterial growth was estimated by determining the protein content by Lowry's method (Lowry *et al.*, 1951).

EXPERIMENTAL FINDINGS AND DISCUSSION

Studies were conducted for the degradation of phenol to use the compound both as carbon and energy source by 10 different bacterial isolates under *in vitro* conditions. In the present study, pure cultures of bacteria isolated by enrichment in phenol, followed by serial dilution and pour plate method were grown in minimal medium containing different concentrations of phenol *viz.*, 10, 20, 30, 40 and 50 mgl^{-1} (Table 1-3).

In the present study, the difference in growth of bacterial isolates may be attributed to the nutrient availability (*i.e.* the phenol concentration) in the growth medium. All the 10 cultures showed a lag in growth up to 48 h period, after which an exponential growth was observed at 72 h. Subsequently, the growth of almost all cultures was observed to decline. This trend was observed with all applied concentrations of phenol except 50 mgL^{-1}. At this particular concentration, the growth of cultures increased slowly and attained maximum growth at 96

h (Table 4).

Degradation rates varied at various concentrations of phenol *viz.*, 10, 20, 30, 40, 50 mgL^{-1} during the various time intervals. At 10 mgL^{-1} the rate of biodegradation was very high but as the concentration rised it was observed that biodegradation level gradually decreased and then attained the maximum degradation at highest concentration at 50 mgL^{-1}. Watanabe *et al.* (1998) reported that in case of *Pseudomonas* sp., the population significantly increased (up to 10 fold) after shock loading of phenol (271ppm). Very less degradation at 50 mgL^{-1} of phenol was observed with *Micrococcus* sp., *Pseudomonas* sp. strain PP1, *Amphibacillus* sp., *Moraxella* sp. MP2, *Aquaspirillum* sp., and *Vibrio* sp. during the various time intervals (Reshma, 2008).

At 50 mgL^{-1} phenol concentration was supplied in maximum in the study at 96 hours which could further act as a sole source of carbon which enhanced the growth of microbial strains and were able to degrade completely up to 100% as expressed in percentage as seen in strain PR1(100%) along with *Campylobacter* sp. (99.95%) and *Mesophilobacter*

Table 1 : Biodegradation of bacterial cultures at different concentrations of phenol after 24 hours (%)

Bacterial isolates	10 mgl^{-1}	20 mgl^{-1}	30 mgl^{-1}	40 mgl^{-1}	50 mgl^{-1}
Moraxella sp. strain MP1	10	31.25	32.5	25	55.5
Pseudomanas sp. strain PP2	45	8.75	15.83	18.75	29.5
Campylobacter sp.	25	47.5	36.66	12.5	32.5
Aeromonas sp.	45	8.75	32.5	30.62	29.5
Neisseria sp.	32.5	8.75	22.5	25	32.5
Erwinia sp.	45	23.75	16.66	9.37	19.5
PR1	12.5	58.75	55.83	21.87	55.5
PR2	5	23.75	50	24.37	32.15
E.coli strain EP2	45	25	32.5	30.62	32.5
Mesophilobacter sp.	5	50	16.66	21.87	55.5

Table 2 : Biodegradation of bacterial cultures at different concentrations of phenol after 48 hours (%)

Bacterial isolates	10 mgl^{-1}	20 mgl^{-1}	30 mgl^{-1}	40 mgl^{-1}	50 mgl^{-1}
Moraxella sp. strain MP1	31.5	56.25	50	49.37	62
Pseudomanas sp. strain PP2	47.5	33.75	29.16	63.75	32.5
Campylobacter sp.	37.5	56.25	58.33	25	68.5
Aeromonas sp.	50	25	63.33	46.87	77.5
Neisseria sp.	47.5	30	63.33	36.87	74.5
Erwinia sp.	50	23.75	24.16	34.37	29.5
PR1	40	73.75	78.33	46.87	84
PR2	32.5	31.25	58.33	36.87	89
E.coli strain EP2	37.5	58.75	55.83	47.5	32.5
Mesophilobacter sp.	25	68.75	50	34.37	91.5

Table 3 : Biodegradation of bacterial cultures at different concentrations of phenol after 72 hours (%)

Bacterial isolates	10 mgl^{-1}	20 mgl^{-1}	30 mgl^{-1}	40 mgl^{-1}	50 mgl^{-1}
Moraxella sp. strain MP1	62.5	68.75	58.33	59.37	79.5
Pseudomanas sp. strain PP2	65	86.25	70.83	68.75	62
Campylobacter sp.	62.5	78.75	70	56.25	72
Aeromonas sp.	50	71.25	80	75	84
Neisseria sp.	55	58.75	81.66	62.5	89.5
Erwinia sp.	80	96.2	55.83	62.5	68.5
PR1	62.5	93.75	93.33	68.75	94
PR2	72.5	75	78.33	48.75	89
E.coli strain EP2	70	73.75	65.83	79.37	62.5
Mesophilobacter sp.	72.5	73.75	60.83	74.37	93.75

Table 4 : Biodegradation of bacterial cultures at different concentrations of phenol after 96 hours (%)

Bacterial isolates	10 mgl^{-1}	20 mgl^{-1}	30 mgl^{-1}	40 mgl^{-1}	50 mgl^{-1}
Moraxella sp. strain MP1	100	77.5	78.33	71.25	98.74
Pseudomanas sp. strain PP2	100	94.2	90	87.5	96.4
Campylobacter sp.	100	100	93.5	87.5	99.95
Aeromonas sp.	100	100	100	100	96
Neisseria sp.	100	100	100	97.5	94
Erwinia sp.	100	97.5	65	85	93.8
PR1	100	100	100	97.5	100
PR2	99.75	97.5	100	85	95
E.coli strain EP2	99.625	100	81.66	80.75	82
Mesophilobacter sp.	100	100	98.33	96.25	99.32

(99.32%), respectively along with other strains which also showed considerable degradation. Biodegradation of phenol by bacterial isolates increased with time period. The maximum degradation was observed at 96 h for all the cultures. In this process, those organisms which are able to survive in the existing environmental conditions and compete most effectively for the available food will remain selected as a biological community.

Conclusion :

Phenol is a common toxic pollutant that can be found in many industrial effluents. From the present study, it is evident that coir-retting environment harbours a variey of potential microbes which can degrade phenols. The growth and adaptability of these bacteria in environment containing phenolic compounds have induced their biodegradation potential. The cultures utilized the substrate as sole carbon source and energy source for their growth and sustenance as an inverse relation was observed between utilization of phenol and percentage degradation in its concentration with increase in the growth of various bacterial isolates *in vitro*.

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