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Quantification of polyphenols during retting and characterization of bacteria from the Kadinamkulam Backwaters, Kerala

Author Details

J.K. Reshma (*Corresponding author*)
Department of Environmental Sciences, All Saints College, Thiruvananthapuram - 695 581, India
e-mail: reshmajk_anand@yahoomail.com

V. Salom Gnana Thanga
Department of Environmental Sciences, University of Kerala - 965 581, India

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Abstract

The retting environment which provides a competitive niche for specialized microbes is speculated to harbour a variety of microbes with high biodegradation potential. In this context, an effort has been made to isolate and identify bacterial species having high tolerance to phenol *In vitro*. Maximum polyphenol (1.897 mg l^{-1}) as observed during the initial period of retting, which decreased as retting proceeded. Based on biochemical characterization, the isolated bacterial strains were identified as *Micrococcus* sp., *Moraxella* sp. strain MP1, *Moraxella* sp. strain MP2 and *Moraxella* sp. strain MP3, *Pseudomonas* sp. strain PP1 and *Pseudomonas* sp. strain PP2, *Amphibacillus* sp., *Brucella* sp. strain BP1 and *Brucella* sp. strain BP2, *Aquaspirillum* sp., *Escherichia coli* strain EP1 and *Escherichia coli* strain EP2, *Campylobacter* sp., *Aeromonas* sp., *Neisseria* sp., *Vibrio* sp., *Erwinia* sp. and *Mesophilobacter* sp. These strains were found to tolerate maximum concentration of phenol *viz.* 200 to 1000 mg l^{-1}. Plasmid analysis of phenol resistant bacterial isolates showed that almost all the cultures had at least one plasmid of size >1Kb. Studies on the protein profile of isolated bacterial cultures showed the presence of proteins with molecular sizes ranging from 10 to 85 KDa with exception of Mesophilobacter and Neisseria having still high molecular weight protein (95 KDa). Bacterial strains isolated from coir-ret-liquor showed tolerance to high phenol concentration.

Key words

Retting, Polyphenols, Bacterial strains, Phenol tolerance, Coir-ret-liquor

Introduction

Polyphenols are a group of chemical substances found in plants, characterized by the presence of more than one phenol group per molecule. Polyphenols are grouped and classified by the type and number of phenolic subcomponents present. More than one subcomponent can be present on a given polypheonol. The subdivision of polyphenols into tannins, lignins and flavanoids is derived from a variety of simple polyphenolic units exclusively from secondary plant metabolism of the shikimate pathway (Matuschek and Svanberg, 2002). Polyphenols come mostly from lignin during its biodegradation and plays a key role in the formation of humic substances (Van Schie and Young, 1998; Arts *et al*., 2005). In addition, they may also enter the environment as intermediates during the biodegradation of xenobiotic compounds. Many of these compounds are resistant to biodegradation.

Phenol pollution is associated with pulp mills, coal mines, refineries, beverage industries, tannery and textile industries, wood preservation plants and various chemical industries, as well as their wastewaters. Phenolics and phthalates are the two common hazardous organic compounds reported to occur in highly organic and heterogenous municipal solid waste typical of an urban scenario in a tropical country (Swati and Joseph, 2007). The coconut husk retting process also liberates large quantities of phenolic compounds in the water column, causing toxic effects to aquatic flora and fauna. According to Jayashankar and Bhat (1966), polyphenols are major constituents, representing as much as 75-76 g kg^{-1} of husk material.

Dead leaves, woody debris, animal remains etc constitute the main sources of organic matter in the backwater environment. Microbial degradation is an important route for removal of these compounds from the environment. Nambiar and Raveendran(2009) studied the diversity of marine fungi from the backwaters of Kerala and isolated 20 marine fungi. Quantification of phenolic compounds during different periods of retting and isolation of bacterial cultures which are capable of phenol bioremediation are highly warranting. This paper is on quantification of phenolic compounds during different

periods of retting and isolation of bacterial cultures which are capable of phenol bioremediation. This is followed by biochemical and molecular characterization of the isolates.

Materials and Methods

Estimation of polyphenols: Estimation of polyphenols was carried out in extract of bacterial culture following the method of Bray and Thorpe (1954). To 1 ml of sample (extract), 2 ml of 20% Na_2CO_3 and 1 ml of diluted Folin-Ciocalteaeu reagent (1:1) were added. The tubes were placed in boiling water bath for 1 minute. Blanks were also treated similarly. Absorbance was measured at 725 nm. The amount of polyphenols in the extract was measured using a standard graph prepared from catechol. The data were analysed statistically as per Snedcor and Cochran (1967).

Sample collection and preparation of bacterial enrichment: Coir ret liquor samples were collected from the retting zone in sterilized screw capped tubes, brought to the laboratory and refrigerated. Enrichments were prepared in nutrient agar added with 50 mg l^{-1} of phenol. Bacteria were isolated using serial dilution and pour plate technique in selective media (Sorbitol agar). Individual colonies showing distinct morphology were selected and purified by further sub culturing. The purified bacterial strains were subjected to phenol tolerance test.

Phenol tolerence test: Pure cultures were grown in selective media (Sorbitol agar). 24 hr old cultures were transfered to minimal medium added with different concentration of phenol (200, 400, 600, 800 and 1000 mgl^{-1}). A control was maintained without phenol. Growth was recorded based on cell OD at A_{600} (Reshma *et al*., 2007). Based on the phenol tolerence test, twenty bacterial cultures were selected for biochemical characterization as per Bergeys Manual of Determinative Bacteriology (1994) and Cappucino and Sherman (1999, 2004) . Plasmid DNA from the bacterial species isolated from the retting zone was extracted as per the method of O' Sullivan and Klenhanner (1993). The bacterial pellet was suspended in 25% sucrose containing 30 µg ml^{-1} lysozyme followed by the addition of 3% alkaline Sodium dodecyl sulphate, ice cold 3 M sodium acetate (pH 4.8) and then isopropanol for precipitation. The resultant pellet was resuspended in water and then 7.5 M ammonium acetate containing 0.5 mg ml^{-1} ethidium bromide and phenol/chloroform (1:1) was added. The DNA from the aqueous phase was precipitated by the addition of ethanol. The pellet was washed and dissolved in Tris-EDTA (Ethylene diamine tetra acetic acid) containing RNAse. The plasmids were resolved by 0.7% agarose gel using TBE buffer at 50 V for 6 hr. Ethidium bromide (0.5 µg ml^{-1}) stained gels were photographed under a U.V. transilluminator.

For genomic DNA isolation (Sambrook, 1989), bacterial cells (24 hr old) were harvested, pelleted and suspended in 4 ml of lysis buffer [15% sucrose, 0.05 M Tris (pH 8.0), 0.05 M EDTA]. SDS was added to final concentration of 1% and then one volume of sterile water and 0.4 mg of proteinase K ml^{-1} were added. The solution was gently mixed and further incubated for 1 hr at 50°C. Briefly, 2 volumes of Tris buffer (pH 8.0) saturated phenol were added and the mixture was gently combined by inverting the tubes until an emulsion was formed. The solution was centrifuged and the aqueous phase was transferred to a clean tube, and the procedure was repeated with phenol-chloroform-isoamyl alcohol (25:24:1) until a clear interface was observed. A final extraction with chloroform-isoamyl alcohol (24:1) was performed. The aqueous phase was then transferred to a clean tube, and the DNA was precipitated with 0.25 volume of 10 M ammonium acetate and 0.6 volumes of isopropyl alcohol at room temperature (28±2°C). The solution was centrifuged and the pellets were washed with 70% ethanol, allowed to dry, and treated for 1 hr at 37°C with RNAse buffer, consisting of 10 mg of RNAse per ml of TE (0.01 M Tris [pH 8], 0.001 M EDTA). DNA was extracted once with phenol-chloroform and again with chloroform-isoamyl alcohol. The nucleic acids were precipitated with 0.1 ml of 3 M sodium acetate (pH 5.2) and two volumes of 95% cold ethanol, washed with 70% ethanol, and dissolved in a small volume of TE. This procedure was repeated for a minimum of two times for each strain used. In addition, samples from each DNA preparation were electrophoresed in 0.8% agarose gels containing ethidium bromide, visualized under a UV transilluminator and photographed.

Results and Discussion

Polyphenol concentration: The concentration of polyphenol released during different periods of retting along the vertical stratification of coir bed is provided in Fig. 1a,b,c. The polyphenol concentration ranged from 0.0003 to 1.897 mgl^{-1}. As per ISI, the permissible limit for phenolic compounds (as C_6H_5OH) for inland surface waters subjected to pollution is 0.002 mgl^{-1} (MoEF, 2000).

The polyphenol concentration of coir-ret-liquor during initial period of retting ranged from 0.35 to 1.737 (avg. 1.21; SD 0.46) in the top layer; 0.079 to 1.808 (avg. 1.21; SD 0.54) in the middle layer and 0 to 1.897 (avg. 1.05; SD 0.62) in the bottom layer. During the middle period of retting, the polyphenol concentration ranged from 0.006 to 0.352 (avg. 0.03; SD 0.09) in the top layer; 0.005 to 0.35 (avg. 0.04; SD 0.09) in the middle layer and 0.007 to 0.036 (avg. 0.02; SD 0.01) in the bottom layer. Towards the end of retting, the polyphenol concentration ranged from 0.0118 to 0.038 (avg. 0.02; SD 0.01) in the top layer; 0.013 to 0.041 (avg. 0.02; SD 0.01) in the middle layer and 0.013 to 0.039 (avg. 0.02; SD 0.01) in the bottom layer.

Paulmurugan *et al.* (2004) reported elevated concentration of phenol in the retting zones (0.47 mgl^{-1}) compared to non retting zones (0 to 0.11 mgl^{-1}). Sediment samples from three stations in mangrove swamps along the Mandovi-Zuari estuarine complex showed the presence of phenolic compounds varying from 0.26 to 1.01 mg kg^{-1}. Phenolic acids such as ferulic, p-coumaric, p-hydroxy benzoic and gentisic were identified by Gomes and Mavinkurve (1982) from the Mandovi-Zuari estuarine complex. Deepa (2001) reported the presence of phenolic compounds namely resorcinol, catechol and phenol are present in the coir fibre steep liquor.

Characterization of bacterial cultures: Based on biochemical characterization (Table 1), the isolated bacterial strains were

Table - 1: Biochemical tests for identification of bacterial strains isolated from coir-ret-liquor

Strains	Biochemical tests															
	Grams staining	Oxidase test	Catalase test	Citrate utilisation	Methyl red	Voges proskauer	Indole test	Motility	Urea hydrolysis	Gelatin hydrolysis	Surcose	Maltose	Dextrose	Lactose	Mannitol	Gas production
Micrococcus	+Cocci	+	+	-	-	-	-	+	-	+	-	-	-	+	+	+
Moraxella	-Cocci	+	+	-	-	-	-	-	-	-	-	+	-	+	-	-
Pseudomonas	-Rod	+	-	-	-	-	+	+	-	+	-	-	-	+	+	-
Amphibacillus	+Rod	+	-	-	-	-	+	+	-	-	-	-	-	+	+	+
Brucella	-Cocci	+	+	-	-	-	-	-	-	+	-	+	-	+	-	-
Moraxella	-Cocci	+	+	-	+	-	-	-	-	-	-	+	-	+	-	+
Pseudomonas	-Rod	+	-	-	-	-	+	+	-	-	+	-	-	+	+	+
Aquaspirillum	-Cocci	+	-	-	-	-	-	-	-	+	-	-	-	+	+	+
E.coli	-Rod	-	+	-	-	-	+	-	+	+	-	-	-	+	-	+
Campylobacter	-Cocci	+	-	-	-	-	+	+	-	+	-	-	-	+	+	+
Aeromonas	-Rod	+	-	-	-	-	-	+	-	+	+	-	-	+	+	+
Neisseria	-Cocci	+	+	-	-	+	+	_	-	+	-	+	-	+	-	+
Vibrio	-Rod	+	+	-	-	-	+	-	-	+	+	+	-	+	+	-
Erwinia	-Rod	-	+	-	-	-	-	-	-	+	+	+	-	+	+	-
PR1	-Cocci	+	-	+	-	+	+	-	-	+	-	+	-	+	-	-
PR2	-Cocci	+	-	-	-	-	+	-	-	+	-	+	+	+	+	+
Brucella	-Cocci	+	+	-	-	-	-	-	+	+	-	-	-	+	+	+
E.coli	-Rod	+	-	-	-	-	+	+	-	-	-	-	-	+	+	+
Mesophilobacter	-Cocci	+	+	-	-	-	+	-	-	-	-	-	-	+	-	+
Moraxella	-Cocci	+	+	+	-	-	-	-	-	-	-	+	-	+	-	+

Table - 2: Tolerance of bacterial culture to different concentrations of phenol (A600 at 24 hr)

Bacterial cultures	Concentration of phenol (mg l^{-1})					
	200	400	600	800	1000	Control
Micrococcus sp.	0.018	0	0.002	0.001	0	0.617
Moraxella sp. strain MP1	0.012	0	0.011	0.004	0.001	0.906
Pseudomonas sp. strain PP1	0.123	0.003	0.009	0.015	0.004	1.084
Amphibacillus sp.	0.023	0	0.004	0.001	0.002	0.408
Brucella sp. strain BP1	0.065	0.003	0.008	0.006	0.003	1.083
Moraxella sp. strain MP2	0.011	0.005	0.004	0.007	0.005	0.484
Pseudomanas sp. strain PP2	0.009	0.009	0.009	0.009	0.008	0.713
Aquaspirillum sp.	0.038	0	0.007	0.006	0	0.345
E.coli sp. strain EP1	0.006	0.001	0.003	0.004	0	0.463
Campylobacter sp.	0.008	0	0	0	0	0
Aeromonas sp.	0.005	0.002	0.001	0.004	0	1.398
Neisseria sp.	0.081	0	0	0.004	0	1.528
Vibrio sp.	0.065	0	0.002	0.001	0	0.963
Erwinia sp.	0.003	0	0	0	0	0
PR1	0.028	0.004	0.004	0.002	0.003	1.587
PR2	0.023	0.004	0.006	0.013	0.013	1.240
Brucella sp. strain BP2	0.068	0.003	0.006	0.025	0.007	1.579
E.coli sp. strain EP2	0.035	0.019	0.009	0.003	0.013	1.140
Mesophilobacter sp.	0.028	0.005	0.013	0.001	0.007	0.361

identified as *Micrococcus* sp., *Moraxella* sp. strain MP1, *Moraxella* sp. strain MP2 and *Moraxella* sp. strain MP3 , *Pseudomonas* sp. strain PP1 and *Pseudomonas* sp. strain PP2 , *Amphibacillus* sp., *Brucella* sp. strain BP1 and *Brucella* sp. strain BP2, *Aquaspirillum* sp., *Escherichia coli* strain EP1 and *Escherichia coli* strain EP2 , *Campylobacter* sp., *Aeromonas* sp., *Neisseria* sp., *Vibrio* sp., *Erwinia* sp. and *Mesophilobacter* sp. Previously Reshma *et al*. (2004; 2007) isolated *E.coli*, *Bacillus*, *Pseudomonas* and

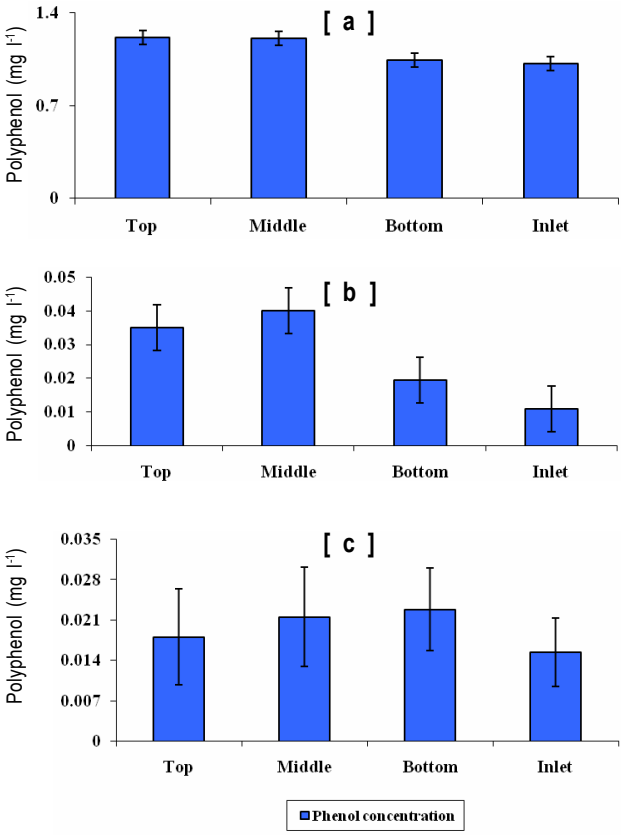
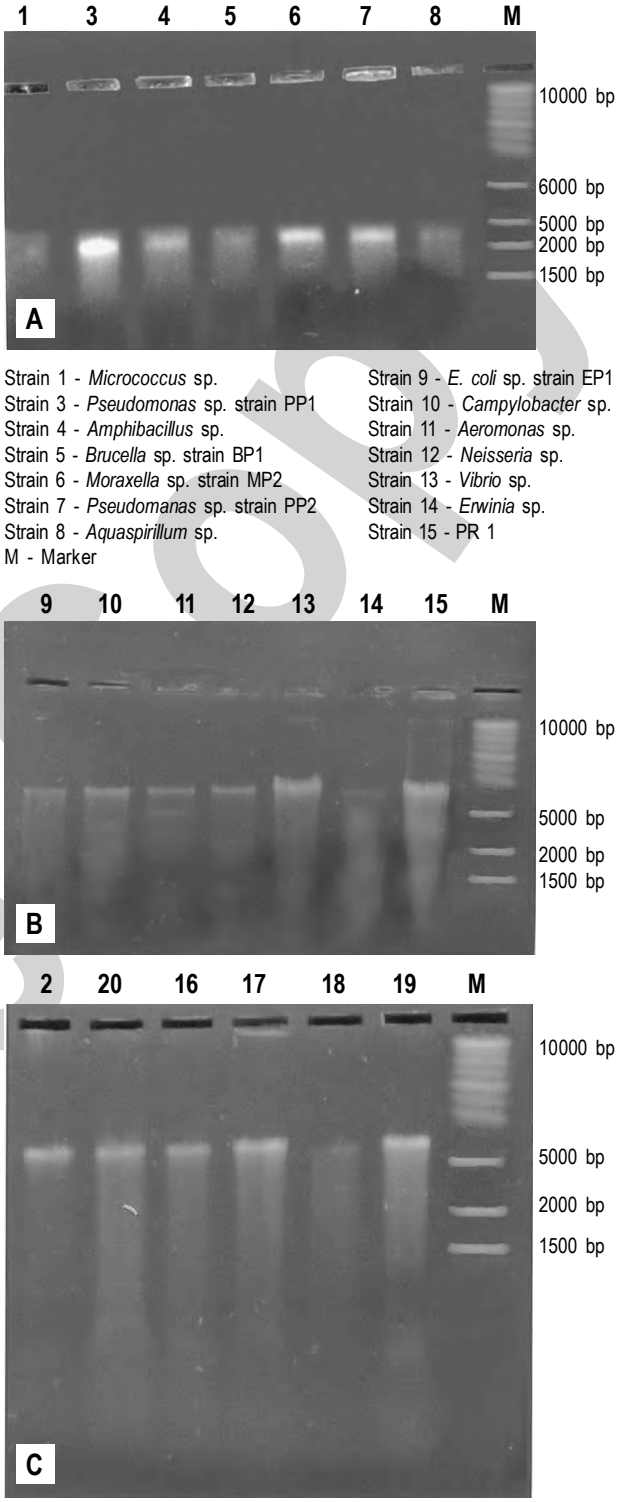


Fig. 1: Polyphenol concentration at different periods of retting along vertical stratification of coir bed (a-initial, b-middle, c-final)

Micrococcus from coir-ret-liquor of Anchuthengu backwaters. Jayashankar and Bhat (1966), from phenol enrichments of coir rets, observed *Pseudomonas* sp. and *Micrococcus* sp. as the specific microflora associated with the leaching of polyphenols. Aresta *et al*. (2010) isolated 28 bacterial strains capable of degrading polyphenols, which were able to use phenol, catechol, caffeic acid and ferulic acid with efficiency ranging from 76 to 95%. The isolated strains were classified belonging to *Arthrobacter sulfurous*, *Pseudomonas synxantha* and *Pseudomonas oryzihabitans*.

Plasmid analysis of phenol resistant bacterial isolates showed that almost all the cultures had at least one plasmid of size > 1Kb (Fig. 2 a,b,c). Certain bacterial cultures *viz., Pseudomonas* sp. strain PP2, *Aeromonas, Campylobacter, Neisseria*, and strain PR1 had additional plasmids. Plasmid profile of the cultures showed presence of a megaplasmid in *Vibrio* and strain PR1. Study on plasmid profile of *Pseudomonas* showed that it had one megaplasmid (approx. 40 MDa) and three plasmids of different sizes (approx. 20, 7 and 4 MDa) (Paulmurugan *et al*., 2004). This may have the genes which can resist the action of pollutants liberated into the medium by husk retting.

Tolerance of bacteria to phenol: Studies were conducted to assess the minimum inhibitory concentration of phenol to the isolated



Strain 1 - *Micrococcus* sp.
Strain 3 - *Pseudomonas* sp. strain PP1
Strain 4 - *Amphibacillus* sp.
Strain 5 - *Brucella* sp. strain BP1
Strain 6 - *Moraxella* sp. strain MP2
Strain 7 - *Pseudomanas* sp. strain PP2
Strain 8 - *Aquaspirillum* sp.
Strain 9 - *E. coli* sp. strain EP1
Strain 10 - *Campylobacter* sp.
Strain 11 - *Aeromonas* sp.
Strain 12 - *Neisseria* sp.
Strain 13 - *Vibrio* sp.
Strain 14 - *Erwinia* sp.
Strain 15 - PR 1
M - Marker

Strain 2 - *Moraxella* sp. strain MP1
Strain 16 - PR 2
Strain 17 - *Brucella* sp. strain BP2
Strain 18 - *E. Coli* sp. strain EP2
Strain 19 - *Mesophilobacter* sp.
Strain 20 - *Moraxella* sp. strain MP3
M - Marker

Fig. 2: Plasmid profile of the isolated bacteria (a, b, c)

bacterial strains. Growth of bacteria at various concentrations of phenol *viz*. 200, 400, 600, 800 and 1000 mg l^{-1} after 24 hr is provided in Table 2. The concentration range was chosen based on the studies of Percival and Senior (1998), which indicated that high concentration of phenol (> 1000 mg l^{-1}) has a detrimental effect on phenol catabolism. After 24 hr of incubation, all the cultures showed slight growth when grown in 200 mg l^{-1} of phenol. As the concentration of phenol increased, growths of most cultures were observed to decrease. Gomes and Mavinkurve (1982) observed that most of the heterotrophic bacterial and yeast isolates from mangrove swamps could tolerate and grow at 0.05% concentration of phenol. Amongst the 114 cultures screened, 35 could grow on phenolic concentration as high as 1%.

The maximum growth was observed with *Pseudomonas* sp. strain PP1 at 200 mg l^{-1}, *E. coli* sp. strain EP2 at 400 mg l^{-1}, *Moraxella* sp strain MP3 at 600 mg l^{-1}, *Brucella* sp. strain BP2 at 800 mg l^{-1} and strain PR2 at 1000 mg l^{-1} during 24 hr. An inverse correlation between bacterial growth and phenol concentration was reported by Joseph and Chandrika (2000) in the sediments of mangrove swamps of Cochin, Kerala, India. Phenolic compounds and total plate count ranged from 0.018 to 16.75 mg l^{-1} and 25 to 110 X $10^4 g^{-1}$ respectively and showed distinct seasonal variations. Phenol concentration was highest during monsoon month, when the bacterial abundance was the lowest. The diversity of bacteria and their number were higher when phenol concentration was less in the sediment. The presence of phenolytic bacteria indicate their potential role in reducing the concentration of the phenolic compounds in the coir steep liquor. This would, in turn lead to decreasing the toxicity of phenolic leachates and thereby develop an ecofriendly process for coir production.

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