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RESEARCH ARTICLE

Isolation and Identification of Phenol Degrading Bacteria from Effluent Treatment Plant of Petrochemical Industry in Kerala

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During the last few decades, an array of foreign compounds has been introduced into the environment due to the revolution of industrial field. Micro-organisms plays a major role for saving our environment by degrading xenobiotic compounds and chemicals wastes, which are toxic either in their native form or modified into toxic substances. Phenol is one of the most common toxic environmental pollutants among various substances produced and released into the environment as a result of various industrial activities. Isolation of microbial strain capable of degrading chemical compounds can be achieved from polluted sources, such as soil and water. The increasing phenol and phenol wastes necessitates the screening of bacteria that are able to degrade phenol. The objective of the study was to isolate and identify the phenol degrading bacteria from effluent treatment plant of petrochemical industry. In this study, the effluent samples were collected from effluent treatment plant of petrochemical industry, Kerala and the bacterial strains capable of degrading phenol were isolated. The cultures were subjected to phenol tolerance level in medium containing different concentrations of phenol (200ppm to 1000ppm). Total phenol content and growth rates were observed for 24 to 96 hrs of incubation for all the five selected strains. From this preliminary observation, among the selected, the most potent strains were *Neisseria sp* and *Micrococcus sp*. The effects of temperature and pH on the growth of these strains were also observed. Outcome of this study offer a useful guideline in evaluating potential phenol degraders from the environment which can be isolated and cultured in large scale and can be used for the waste management.

INTRODUCTION

In the development of the world today, human health and the environment have become the most pressing issues. Due to those concerns, biodegradation of aromatic compounds have received a great attention because of their toxicity and persistence in the environment. Among all the toxic compounds, phenol and its substituent phenolic compounds contribute a remarkable adverse impact to the environment. These are major xenobiotics, which are often found in the effluents discharged from the industries such as paper and pulp, textiles, gas and coke, fertilizers, pesticides, steel and oil refineries etc., (Ghadi and Sangodkhar, 1995). During the last two decades, phenolic compounds have become the subject of intense research in the preservation of our environment. Due to the toxic properties, including permeabilisation of cellular membranes and cytoplasm coagulation, phenolic contaminants can damage sensitive cells and cause profound health and environmental problems (Tziotzios *et al*., 2005). The World Health Organization has limited phenol concentration in the water to 1 mg/L (WHO, 1994).

Most of the efficient phenol degrading microorganisms are capable of using phenol as the sole source of carbon and energy for their growth and metabolism. Microorganisms capable of degrading phenol do so with the action of a variety of enzymes. They first convert Phenol to catechol with the help of hydroxylase enzyme. Catechol is then degraded to its intermediates via ortho or Meta cleavage with the help of another set of enzymes. The enzyme catechol 2, 3 dioxygenase cuts the benzene ring of catechol at the Meta position and the enzyme catechol 1, 2 dioxygenase cuts the benzene ring at the ortho position. The intermediates released troughortho and meta cleavage are finally consumed by the microbes with the help of various enzymes through the TCA cycle resulting in CO2, metabolites and energy.(Nair C *et al.*,2008, Agarry S.E and Solomon, 2008, Basha k *et al.*, 2010).

Materials and Methods

Samples from effluent treatment plants of petrochemical industry were collected and serially diluted. Microbial Enrichment was done using nutrient broth with different phenol concentrations (10, 20, 30, 40, 50 ppm). From the 50 ppm culture, organisms were collected and added to sorbitol agar medium with varying concentrations of phenol (200, 400, 600, 800 and 1000 ppm).

Estimation of Total Phenol

Estimation of total phenol was carried out using the method of Bray *et al*., 1954.

Observation of Total Growth

The growth rates of the microbes were observed by spectrophotomeric analysis.

Identification of Isolates

Biochemical Characterization was done based on Bergeys manual of Determinative Bacteriology(1994) and the method prescribed by Cappucino*et al*.,(1999, 2000).

Effect of pH and temperature on total growth and phenol degradation

The effectof pH and temperature on total growth and phenol degradation of the selected strains were studied.

Results and Discussion

One of the most alarming situations in today's world is the generation of a huge amount of waste water contaminated with the toxic organic substances like phenolics from the industrial sector. Phenol is highly water soluble, and its presence in the water imparts a carbolic odor to the receiving water bodies and can have baleful effects on aquatic as well as terrestrial flora and fauna including human beings (ATSDR, 2008). Hence removal of phenol from the discharged sewage and effluent is highly necessary. Conventional methods for treatment of phenol have their own set of disadvantages hence biological method is the current choice. Reports on possibility of treatment of phenol bearing waste water using microorganisms are also available (Nandish, 2005). Till date, research communities have focused only on the isolation of microorganisms from the sites contaminated with phenol industrial effluents and use them for the treatment of the industrial wastewater. Samples from effluent treatment plants of petrochemical industry were chosen as the source of indigenous microorganism's isolation in this study. High probability of the presence of toxic pollutants in this area was reason why site was selected (Nuhoglu and Yalcin, 2004).

Serially diluted samples were transferred to 10,20,30,40 and 50 ppm phenol having minimal media. Neumann *et al.*,(2004) adapted *Pseudomonas* strains to high concentrations of phenol (1000 mg/L) and further biodegradation was carried out at a concentration of 500 mg/L. They opinioned that the cultures could grow by utilizing phenol as a source of carbon and energy. Growth and total phenol was recorded according to its incubation periods. The growth rate and the total phenol content for the microbes were given in the table 2.

Identification of microbial strains

In the samples, from 10 ppm to 50 ppm, total phenol was minimum in 50 ppm concentration of phenol and maximum in 20 ppm. Second most in 10 ppm, then followed by 30 ppm and 40ppm. The least phenol degradation was in 20 ppm. In these all the strains were active upto 72 hrs then gradually decreased during 96 hrs incubation. It has been suggested that several factors affect the growth of bacteria in a microbial community (Roszak and Colwell, 1984); some of these factors are nutrient availability, the presence of toxins, attachment of cells to matrices, and physical parameter. Out of 13 strains isolated, 5 strains were selected for further analysis. These 5 strains were *Neisseria sp*, *Erwinia sp*, *Micrococcus sp*, *Mesophilobacter sp* and *Campylobacter sp* (Table 1 and Figures 1 to 5). All the selected 5 strains were inoculated in sorbitol agar media with different concentrations of phenol (200ppm, 400ppm, 600ppm,

800ppm and 1000ppm). Total phenol content and growth rate were observed for 24 to 96 hrs of incubation and the results are given in the (Tables 3 to7).

The biochemical test of the selected strains, were done using the Bergeys manual of Determinative Bacteriology (1994) and Cappucino*et al*. (1999, 2000). (Table 1 and Figure 1- 5). All the selected strains were -vecocci, except *Erwinia sp*. All the strains except *Erwinia sp* had shown +ve result towards the oxidase test and gas production and all showed –ve result towards the citrate utilization, methyl red and urea hydrolysis tests.

Table 1- Biochemical tests of selected strains.

Tests / Strains	Gram staining	Oxidase test	Catalase test	Citrate utilization	Methyl red	Vogespr oskauer	Indole test	Motality	Urea hydrolysis	Gelatin hydrolysis	Gas production
Neisseria sp	-cocci	+	+	-	-	+	+	-	-	+	+
Erwinia sp	-Rod	-	+	-	-	-	-	-	-	+	-
Micrococcus sp	-cocci	+	+	-	-	-	-	+	-	+	+
Mesophilobacter sp	-cocci	+	+	-	-	-	+	-	-	-	+
Camphylobacter sp	-cocci	+	-	-	-	-	+	+	-	+	+

Table 2- Growth and total phenol of strains

	Growth (600nm)				Total phenol (725nm)			
con.in ppm	24 hrs	48 hrs	72 hrs	96 hrs	24 hrs	48 hrs	72 hrs	96 hrs
10	0.202	0.211	0.198	0.169	0.837	0.757	0.513	0.458
20	0.192	0.201	0.184	0.161	0.794	0.734	0.614	0.544
30	0.193	0.199	0.192	0.189	0.761	0.670	0.530	0.480
40	0.193	0.203	0.190	0.189	0.859	0.700	0.603	0.490
50	0.196	0.217	0.992	0.195	0.749	0.408	0.339	0.272

Strains isolated from the sample (Figure 1 to 5)

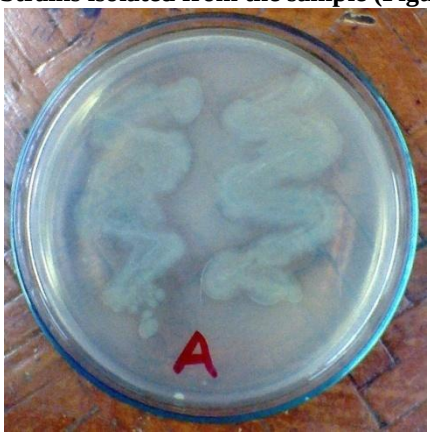

Figure 1 – *Neisseria sp*

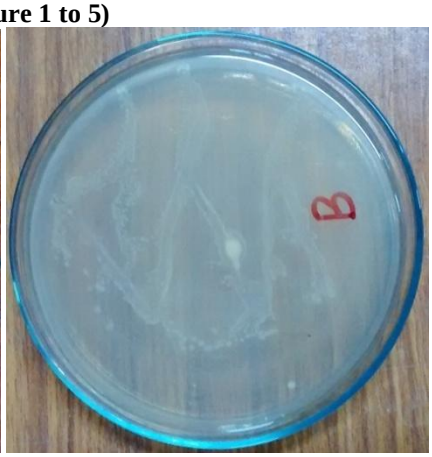

Figure 2-*Erwinia sp*

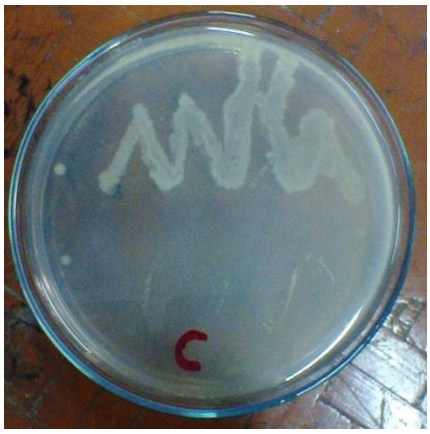

Figure 3 - *Micrococcus sp*

Figure 4- *Mesophilobacter sp*

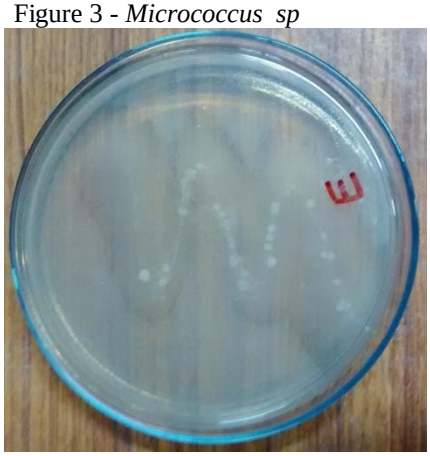

Figure 5- *Campylobacter sp*

In medium with 200 ppm, the highest degradation potential was shown by *Mesophilobacter sp* during 72 hrs incubation and then followed by *Camphyolobacter sp*, *Micrococcus sp*, *Erwinia sp* and *Neisseria sp*. From this it is clear that strain *Neisseria sp* having low degradation capacity in 200 ppm. In 400 ppm, the maximum growth was shown by *Micrococcus sp* then followed by *Erwinia sp*, *Mesophilobacter sp*, *Campylobacter sp* and *Neisseria sp.*
Neisseria sp was having high biodegradation potential in medium with 600 ppm. Then the second most one was *Camphylobacter sp* followed by *Erwinia sp*, *Mesophilobacter sp* and *Micrococcus sp*. In 800ppm phenol of medium, *Micrococcus sp* showed high degradation capacity and least showed by *Erwinia sp* in between *Camphylobacter sp* , *Mesophilobacter sp* and *Neisseria sp*. In medium with highest phenol content of 1000ppm concentration, maximum degradation and acclimatization was shown by *Micrococcus sp* followed by *Mesophilobacter sp*, *Erwinia sp*, *Campylobacter sp* and *Neisseria sp*. Shen and Wang (1993) conducted experiments on simultaneous chromium reduction and phenol degradation in a co-culture of *E coli* and *Pseudomonas putida*. They also reported similar growth pattern with a short lag period, followed by an exponential growth phase and then a declining growth phase.
From those primary observations, it was clear that *Neisseria sp* was having the capacity to thrive well in 400 and 600 ppm. In 200 ppm, *Erwinia sp* showed highest growth. *Micrococcus sp* and *Mesophilobacter sp* having the potential to degrading phenol concentration of 1000 ppm, 800 ppm and 400ppm. *Camphylobacter sp* showed highest survival in 600ppm and 800ppm concentration of phenol. All these strains were active till 72 hrs of incubation after that slight growth decline was observed. Regina *et al*., (2004) studied the behavioral patterns of hydrocarbon utilizing bacteria in media containing different concentrations of crude oil. They showed that lower doses of crude oil were more highly utilized than higher doses. They accounted this for the high growth response in the low dose of crude oil.
The total phenol content was slightly increased after the 48 hrs of incubation and the same time growth was decreased. After that when growth rate increased, total phenol decreased. This was shown by all the concentrations of phenol (by 200-1000ppm) with various strains. In the selected sample, among the selected strains, *Neisseria sp* and *Micrococcus sp* showed the most potential degradation in all the concentrations of phenol.

Table 3- Growth and total phenol (200ppm)

	Growth (600nm)				Total phenol (725nm)			
Strains	24 hrs	48 hrs	72 hrs	96 hrs	24 hrs	48 hrs	72 hrs	96 hrs
Neisseria sp	0.008	0.005	0.008	0.004	0.019	0.114	0.029	0.021
Erwinia sp	0.021	0.004	0.009	0.004	0.045	0.122	0.015	0.015
Micrococcus sp	0.026	0.004	0.007	0.004	0.006	0.134	0.040	0.021
Mesophilobacter sp	0.023	0.004	0.006	0.004	0.101	0.128	0.110	0.035
Camphylobacter sp	0.018	0.004	0.005	0.005	0.125	0.097	0.090	0.090

Table 4- Growth and Total phenol – (400ppm)

	Growth (600nm)				Total phenol (725nm)			
Strains	24 hrs	48 hrs	72 hrs	96 hrs	24 hrs	48 hrs	72 hrs	96 hrs
Neisseria sp	0.005	0.004	0.007	0.005	0.009	0.192	0.066	0.006
Erwinia sp	0.005	0.004	0.004	0.001	0.047	0.198	0.104	0.100
Micrococcus sp	0.007	0.002	0.003	0.001	0.086	0.187	0.140	0.140
Mesophilobacter sp	0.008	0.004	0.006	0.005	0.192	0.197	0.058	0.023
Camphylobacter sp	0.008	0.004	0.005	0.004	0.060	0.201	0.037	0.036

Table 5- Growth and Total phenol – (600ppm)

	Growth (600nm)				Total phenol (725nm)			
Strains	24 hrs	48 hrs	72 hrs	96 hrs	24 hrs	48 hrs	72 hrs	96 hrs
Neisseria sp	0.005	0.004	0.007	0.004	0.078	0.303	0.043	0.022
Erwinia sp	0.004	0.003	0.004	0.002	0.297	0.317	0.269	0.147
Micrococcus sp	0.004	0.001	0.003	0.001	0.302	0.291	0.261	0.181
Mesophilobacter sp	0.005	0.004	0.004	0.002	0.310	0.304	0.171	0.153
Camphylobacter sp	0.004	0.004	0.006	0.005	0.283	0.301	0.151	0.123

Table 6- Growth and Total phenol – (800ppm)

	Growth (600nm)				Total phenol (725nm)			
Strains	24 hrs	48 hrs	72 hrs	96 hrs	24 hrs	48 hrs	72 hrs	96 hrs
Neisseria sp	0.009	0.006	0.007	0.006	0.093	0.189	0.170	0.170
Erwinia sp	0.006	0.003	0.004	0.002	0.104	0.279	0.189	0.180
Micrococcus sp	0.008	0.005	0.008	0.004	0.131	0.256	0.108	0.105
Mesophilobacter sp	0.007	0.006	0.006	0.005	0.156	0.296	0.167	0.151
Camphylobacter sp	0.006	0.004	0.007	0.004	0.106	0.253	0.139	0.123

Table 7- Growth and Total phenol – (1000ppm)

	Growth (600nm)				Total phenol (725nm)			
Strains	24 hrs	48 hrs	72 hrs	96 hrs	24 hrs	48 hrs	72 hrs	96 hrs
Neisseria sp	0.006	0.004	0.006	0.004	0.241	0.350	0.351	0.351
Erwinia sp	0.005	0.004	0.004	0.003	0.290	0.380	0.359	0.304
Micrococcus sp	0.008	0.004	0.004	0.001	0.308	0.400	0.302	0.261
Mesophilobacter sp	0.009	0.003	0.005	0.004	0.304	0.400	0.295	0.290
Camphylobacter sp	0.003	0.003	0.003	0.001	0.271	0.388	0.348	0.333

Effect of pH and Temperature on the selected strains

For the study of effect of pH on the degradation of phenol from the selected strains of the samples, an acidic (pH 5) and basic (pH 9) medium were selected (Table 10 and 11). From this it was clear that when pH decreased, growth also slightly decreased and the degradation did not occur properly. In the case of increasing pH, growth was also increased but degradation rate was very less. In this study, only standard optimum pH had given the accurate phenol degradation correspondingly with their growth. The internal environment of all living cell is believed to be approximately neutral. Most organisms cannot tolerate pH values below 4.0 or above 9.0 (Kim. N.W & Armstrong M.E 1981). At low (4.0) or high (9.0) pH values acids or bases can penetrate into cells more easily, because they tend to exist in undissociated form under these conditions and electrostatic force cannot prevent them from entering cells (Robertson *et al.,* 1992,

Chitra 1995, Annadurai *et al* 1999). The optimum pH for phenol degradation is 7.0 for *Pseudomonas putida*NICM 2174 (Annadurai *et al.*, 2000).

For the analysis of effect of temperature, two optimum temperatures were selected (27^0 C and 47^0 C) (Table 8 and 9). From this study, it was clear that all the selected strains had shown very negligible amount of phenol degradation in low and high temperature. So it can be concluded that the standard prescribed temperature is suitable for better growth and phenol degradation. Temperature plays an important role than nutrient availability in the degradation of organic pollutants. According to Pakula *et al.,* (Pakula .A *et al.*, 1999) phenol biodegradation was significantly inhibited at 30°C. However, most laboratory studies on phenol degradation have been carried out at an optimum temperature of 30°C (Annadurai. G *et a.,l* 2002, Paraskevi N.P *et al.,*2005).Annadurai *et al.,*1999 and Chitra,1995 described that when the temperature increased from 30°C to 34°C,no phenol degradation was observed due to cell decay, which shows that the phenol degradation is a temperature dependent process. Growth rates in general roughly double for each 10°C rise in temperature within the usual mesophilic operational range from 10 to 30°C. Growth rates generally do not change between 35°C and 40°C, but denaturation of proteins at higher temperatures slows growth rates for mesophiles. However, different mixed cultures adapted to thermophilic temperatures have optimum temperatures range of 55 to 65°C. Thermophiles do not function well at the intermediate temperature of 40 to 45°C as mesophilic organisms. Thus, one must make the decision to operate at the lower mesophilic range with an optimum temperature of around 35°C or in the thermophilic range with a temperature optimum of 55 to 60°C.

Table- 8- Growth and Total phenol in Temperature 27 ^{0}C in 72 hrs

Strains	Growth 600nm	Total phenol 720nm
Neisseria sp	0.003	0.400
Erwinia sp	0.004	0.372
Micrococcus sp	0.003	0.391
Mesophilobacter sp	0.002	0.400
Campylobacter sp	0.004	0.400

Table 9- Growth and Total phenol in Temperature 47 ^{0}C in 72 hrs

Strains	Growth 600nm	Total phenol 720nm
Neisseria sp	0.009	0.400
Erwinia sp	0.009	0.400
Micrococcus sp	0.005	0.400
Mesophilobacter sp	0.005	0.400
Campylobacter sp	0.006	0.400

Table 10 - Growth and Total phenol in pH 5 in 72 hrs (800ppm)

Strains	Growth 600nm	Total phenol 720nm
Neisseria sp	0.003	0.400
Erwinia sp	0.003	0.363
Micrococcus sp	0.005	0.400
Mesophilobacter sp	0.003	0.400
Campylobacter sp	0.004	0.400

Table- 11- Growth and Total phenol in pH 9 in 72 hrs (800ppm)

Strains	Growth 600nm	Total phenol 720nm
Neisseria sp	0.003	0.400
Erwinia sp	0.010	0.312
Micrococcus sp	0.004	0.400
Mesophilobacter sp	0.003	0.400
Campylobacter sp	0.006	0.400

Combination of microbes for biodegradation

In order to find out and compare the efficiency of consortium and individual organisms, the medium with 800 ppm was selected and used for total growth and total phenol studies. When consortium of the organisam was introduced in nutrient broth of 800 ppm concentration, it showed a rapid degradation according to the growth. After 48 hrs of incubation they expressed less degradation rate (Table 12). This study revealed that introducing individual organism had shown the maximum degradation of phenol rate than the rate of consortium of organism degradation rate. Evolution of efficient degradative pathways is also likely to be slow when degradation is accomplished by co-metabolism by a consortium of microbes (Shelley D., 2009). For example, several microbes catalyze transformations of TNT via co-metabolic processes that reduce the nitro substituent, or less commonly, remove a nitro substituent. However, microbe carries out enough reactions to derive a benefit from its degradation. Evolution of an efficient degradative pathway may be more rapid when several consecutive reactions, or even the entire pathway, are found within a single microbe that can reap the advantages of accessing a novel source of carbon, nitrogen, or phosphorous.

Table-12- Growth and Total phenol of Consortium of bacteria (800 ppm)

Effluent	24 hrs	48 hrs	72 hrs	96 hrs
Growth(600 nm)	0.141	0.157	0.025	0.017
Total phenol(720 nm)	0.039	0.400	0.307	0.209

Summary and Conclusion

With the immense growth of industries, major problem is encountered as contamination of the environment with hazardous and toxic chemicals. Phenolics, one of the major pollutants, are discharged in the waste water from the various industries such as phenolic resin and pharmaceuticals, oil refineries, petrochemical plants, textile industry, ceramic plants, steel plants, and coal conversion processes. Effluent of petrochemical industry is the most potential source to isolate high performance phenol degrading microorganisms. The degradation performance of selected strains was examined for different phenol concentrations. Serial exposure to increasing level of phenol concentration was used to determine the resistance of isolated strain. Acclimatization of the microorganisms overcomes the substrate inhibition problems that normally occurred in phenol biodegradation at high concentration (Lob and Tar, 2000). The experiment aimed to find the highest tolerance level of phenol concentrations and found that it was able to survive and degrade up to 800 mg/L phenol. The growth of bacteria and phenol concentration in the media showed the inverse proportion with each other. The decrease in phenol concentration accompanied with increase in biomass. From the preliminary analysis, among the selected strains, the most potent ones were *Neisseria sp and Micrococcus sp*. They showed high degradation potential in most of the higher phenol concentrations. The results of study on effects of pH and temperature revealed that the standard optimum pH and temperature were needed for normal bacterial growth and phenol consumption as their energy source. This study revealed that introducing individual organism gave the maximum degradation of phenol rate than the rate of consortium of organism.

Serial exposure to increasing level of phenol concentration can be used to determine acclimatizability of a particular isolate. Highly acclimatizable microbes are those which are able to degrade phenol at high concentration and at greatest rate will be the best phenol degrader candidates. Future studies should be carried out to analyze the best phenol degrading bacteria from the effluents of various samples.

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