ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 8, Issue, 03, pp. 27173-27178, March, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

ISOLATION OF GENOMIC AND PLASMID DNA OF SELECTED PHENOL DEGRADING BACTERIA FROM INDUSTRIAL EFFLUENT TREATMENT PLANTS

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ARTICLE INFO

Article History:
Received 09th December, 2015
Received in revised form
20th January, 2016
Accepted 28th February, 2016
Published online 16th March, 2016

Key words:

Phenol degradation,
Industrial effluent,
Neisseria sp,
Micrococcus sp,
Psuedomonas sp,
Genomic DNA,
Plasmid DNA.

ABSTRACT

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, ceramic plants, steel plants, and coal conversion processes. 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. The main objective of this study was to isolate the genomic and plasmid DNA from selected potent phenol degrading bacteria from the effluent treatment plants of textile and petrochemical industries. Total 10 strains were isolated from both the samples and out of that four potent strains from both the samples were used for the isolation of genomic and plasmid DNA. They were *Neisseria* sp and *Micrococcus* sp from petrochemical effluent and *Micrococcus* sp and *Pseudomonas* sp from textile effluent as they are found to be the most potent among the selected strains. They gave maximum degradation potential in the medium with 800 ppm phenol. From the results, it was clear that strain 1 contained a clear band of 700 bp. Strain 2, 3 and 4 showed a feeble band at 1200 bp position. The plasmid DNA content was relatively less in 3 strains and almost absent in one strain. As a future perspective, the plasmid DNA can be removed from the strains and those without plasmid can be used to study the degradation potential of selected strains using the same effluent so as to investigate the plasmid mediated degradation.

Citation: Rajani, V. and Reshma, J.K. 2016. "Isolation of genomic and plasmid DNA of selected phenol degrading bacteria from industrial effluent treatment Plants", *International Journal of Current Research,* 8, (03), 27173-27178.

INTRODUCTION

Environmental pollution is considered as a side effect of modern industrial society. 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 phenol resin and pharmaceutical, oil refineries, petrochemical plants, ceramic plants, steel plants, and coal conversion processes. Phenol and its derivatives is the basic structural unit in a wide variety of synthetic organic compounds (Annadurai *et al*., 2000). Phenol and its higher homology are aromatic molecules containing hydroxyl group attached to the benzene ring structure. The focus on the microbial degradation of phenols in recent years has resulted in the isolation, culture, adaptation and enrichment of a number of microorganisms that can grow on the compound as a sole carbon and energy source. Phenol is an antimicrobial agent; many of the microbes are susceptible to this compound. However, there are some microbes, which are resistant to phenol and have the ability to degrade phenol. The wide varieties of microorganisms that can aerobically degrade phenol include pure bacterial cultures. Biological treatment is one of the considerable choices for removing of phenol present in these wastewaters. Identification of effective microbial species is considered as one of the important priorities for production of the biomass in order to achieve desirable kinetic of biological reactions. The ability of microorganisms to degrade an organic compound is the result ultimately of the genetic makeup of the organisms. The chemical reactions involved in metabolism are mediated by enzymes. The range of enzymes which a bacterium has is a reflection of the specific genetic information in the cell. Genetic information in bacteria, as in all organisms, is stored in the form of DNA. The information is physically present in bacterial cells in two forms – the chromosome and the plasmids. The bacterial chromosome is a single circular, highly folded double-strand of DNA. In addition to chromosomal DNA, a larger number of bacteria

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also have extra-chromosomal DNA in the form of plasmids (Zylstra and Gibson, 1991). The main objective of the study was to identify the potent phenol degrading bacteria from the effluent treatment plants and to isolate the genomic and plasmid DNA of the selected strains.

MATERIALS AND METHODS

Sample collection- The samples were collected from effluent treatment plants of textile and petrochemical industries. From both the sites samples were collected in sterilized screw top sterile bottles and brought to the lab and kept in refrigerator till use.

Serial dilution- Serial dilution of the collected samples was carried out using sterile distilled water.

Enrichment of microorganism- Microbial Enrichment was done using nutrient broth containing different phenol concentrations (10, 20, 30, 40 and 50 ppm). From the 50 ppm culture, organisms were collected and added to selective media called sorbitol agar media.

Biochemical characterization- From the observed colonies, a total of 10 strains were selected, 5 each from both the samples for further biochemical characterization. Biochemical characterization of these bacterial strains was carried out based on Bergeys manual of Determinative Bacteriology (1994) and Cappucino *et al*., (1999).

Phenol Determination- Phenol determination was done using method of Bray *et al*., 1954.

Growth Determination- The growth determination was done by measuring the absorbance at 600 nm using UV-Vis spectrophotometer.

Genomic DNA isolation- Total genomic DNA was isolated using Purelink Genomic DNA isolation kit (Invitrogen, USA) as per manufacturer's description.

Plasmid DNA isolation- Plasmid DNA was isolated using Purelink quick plasmid isolation kit (Invitrogen, USA).

RESULTS AND DISCUSSION

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 tables 1and 2. In the petrochemical effluent 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 up to 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. 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. Out of 11 strains isolated from textile effluent, 5 strains were selected for further analysis. From these also 5 strains were selected for further studies. They are *Micrococcus* sp, *Brucella* sp, *Pseudomonas* sp*, Aquaspirillum* sp and *Moraxella* sp. All the 5 strains were inoculated in sorbitol agar media with different concentrations of phenol (200ppm, 400ppm, 600ppm, 800ppm and 1000ppm). Total phenol content and growth rates were observed for 24 to 96 hrs of incubation.

Identification of microbial strains

The biochemical test of the selected strains, were done using the Bergeys manual of Determinative Bacteriology (1994) and Cappucino *et al*. (1999, 2000) (Table 3 and 4). All the selected strains from petrochemical plant were -ve cocci, 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. 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 showed maximum biodegradation potential of medium with 600 ppm. 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 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 *Escherium 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. In medium with 200 ppm, the maximum degradation was shown by *Aquaspirillum* sp followed by *Pseudomonas* sp, *Moraxella* sp*, Micrococcus* sp and the least one was *Brucella* sp. The *Micrococcus* sp having the highest growth in 400ppm concentration of phenol, followed by *Moraxella* sp*., Brucella* sp*., Aquaspirillum* sp. and *Pseudomonas* sp. In 600 ppm, the maximum degradation potential had shown by *Pseudomonas* sp and the second most

was *Moraxella* sp followed by *Pseudomonas* sp*, Micrococcus* sp and *Aquaspirillum* sp. In case of textile effluent, in the medium with 800 ppm of phenol, the highest degradation potential was shown by *Micrococcus* sp followed by *Pseudomonas* sp, *Aquaspirillum* sp, *Moraxella* sp and the least one was by *Brucella* sp. In 1000ppm concentration of phenol, maximum growth and phenol degradation were shown by *Pseudomonas* sp followed by *Moraxella* sp, *Aquaspirillum* sp*, Micrococcus* sp and *Brucella* sp. Bandyopadhyay *et al*. (1998) had studied the biodegradation of phenol by *Pseudomonas putida* immobilized in calcium alginate and reported that on increasing the concentration of the phenol above 750 ppm the reaction behavior deviates from michelean kinetics which may be due to the effect of intra-particle diffusion. From the analysis it was clear that *Micrococcus* sp was having the potential to survive in 400ppm, 600ppm and 800ppm concentrations of phenol. *Brucella* sp was actively found in 400ppm and 600ppm concentrations of phenol. *Pseudomonas* sp thrive well in 600ppm, 800ppm and 1000ppm concentrations of phenol and *Aquaspirillum* sp showed highest growth in 200 ppm concentration of phenol. *Moraxella* sp showed highest degradation potential in 400 ppm.

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 is 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, *Neisseria* sp and *Micrococcus* sp showed most potential degradation in all the concentrations of phenol (tables 5 and 6).

Isolation of Genomic and Plasmid DNA

From the 10 selected strains from both the industrial effluents, 2 potent strains from each sample were used for the isolation of genomic and plasmid DNA. They were *Neisseria* sp and *Micrococcus* sp from petrochemical effluent and *Micrococcus* sp and *Pseudomonas* sp from textile effluent. They gave maximum degradation potential in the medium with 800 ppm phenol (Tables 5 and 6). The ability of microorganisms to degrade an organic compound is the result ultimately of the genetic makeup of the organisms. The chemical reactions involved in metabolism are mediated by enzymes. The range of enzymes which a bacterium has is a reflection of the specific genetic information in the cell.

Table 1. Growth and total phenol of strains from petrochemical effluent

		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

Table 2. Growth and total phenol of strains from textile effluent

		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	0.071	0.126	0.020	0.018	0.908	0.673	0.462	0.318
20	0.068	0.093	0.015	0.008	0.711	0.708	0.483	0.304
30	0.099	0.186	0.019	0.014	0.860	0.634	0.454	0.226
40	0.070	0.145	0.028	0.014	0.732	0.641	0.421	0.416
50	0.091	0.163	0.023	0.013	0.721	0.653	0.443	0.436

The total phenol was slightly increased after the 48 hrs of incubation and at the same time growth was decreased. After that when growth rate increased, total phenol decreased. This was shown in all the concentrations of phenol (by 200-1000ppm) with various strains. Among the selected 5 strains, *Micrococcus* sp and *Pseudomonas* sp were the strains that could withstand all the selected concentrations of phenol. 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

Genetic information in bacteria, as in all organisms, is stored in the form of DNA. The information is physically present in bacterial cells in two forms – the chromosome and the plasmids. The bacterial chromosome is a single circular, highly folded double-strand of DNA. In addition to chromosomal DNA, a larger number of bacteria also have extra-chromosomal DNA in the form of plasmids (Zylstra and Gibson, 1991). Many plasmids contain genes which code for the enzymes necessary for the derivative pathways important to bioremediation. In the study, an attempt was made to isolate the genomic and plasmid DNA (Figures 1 and 2). From the results, it was clear that strain 1 (*Neisseria* sp from petrochemical effluent) contained a clear band of 700 bp. Strain 2 (*Micrococcus* sp from petrochemical effluent), strain 3 (*Micrococcus* sp from textile effluent) and strain 4 (*Pseudomonas* sp from textile effluent) showed a feeble band at 1200 bp position (Figure 1). The plasmid DNA content was relatively less in 3 strains and almost absent in one strain (Figure 2).

Table 3. Biochemical test results of selected strains (petrochemical effluent)

Tests Strains	Gram staining	Oxidase test	Catalase test	Citrate utilization	Methy l red	Voges proskauer	Indole test	Motality	Urea hydrolysis	Gelatin hydrolysis	Gas production
Neisseria sp	-cocci	+	+	-	-	+	+	-	-	+	+
Erwinia sp	-Rod	-	+	-	-	-	-	-	-	+	-
Micrococcus sp	-cocci	+	+	-	-	-	-	+	-	+	+
Mesophilobacter sp	-cocci	+	+	-	-	-	+	-	-	-	+
Campylobacter sp	-cocci	+	-	-	-	-	+	+	-	+	+

Table 4. Biochemical test results of selected strains (Textile effluent)

Tests Strains	Grams staining	Oxidase test	Catalas e test	Citrate utilization	Methyl red	Voges proskauer	Indole test	motality	Urea hydrolysis	Gelatin hydrolysis	Gas production
Micrococcus sp	+cocci	+	+	-	-	-	-	+	-	+	+
Brucella sp	-cocci	+	+	-	-	-	-	-	-	+	-
Pseudomonas sp	-rod	+	-	-	-	-	+	+	-	-	+
Aquaspirillum sp	-cocci	+	-	-	-	-	-	-	-	+	+
Moraxella sp	-cocci	+	+	-	-	-	-	-	-	-	-

Table 5. Growth and Total Phenol - 800ppm (Petrochemical effluent)

Growth (600 nm)						Total	Phenol	(725nm)
Strains/incubation periods	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
Campylobacter sp	0.006	0.004	0.007	0.004	0.106	0.253	0.139	0.123

Table 6. Growth and Total Phenol - 800ppm (Textile effluent)

Growth (600 nm)						Total	Phenol	(725nm)
Strains/incubation periods	24 hrs	48 hrs	72 hrs	96 hrs	24 hrs	48 hrs	72 hrs	96 hrs
Micrococcus sp	0.005	0.004	0.009	0.005	0.153	0.291	0.129	0.108
Brucella sp	0.006	0.002	0.003	0.001	0.141	0.263	0.172	0.170
Pseudomonas sp	0.006	0.004	0.007	0.004	0.135	0.249	0.123	0.119
Aquaspirillum sp	0.006	0.004	0.006	0.004	0.111	0.278	0.139	0.121
Moraxella sp	0.007	0.004	0.005	0.004	0.126	0.281	0.146	0.121

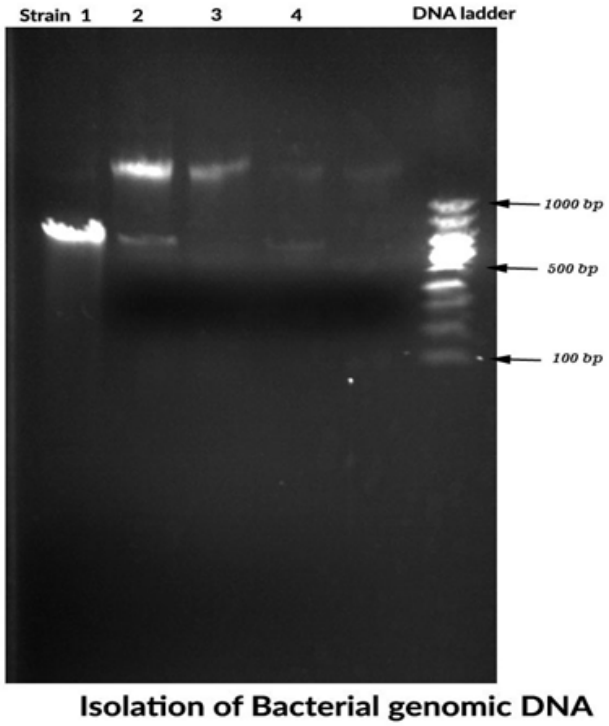

Fig. 1. Isolation of Genomic DNA

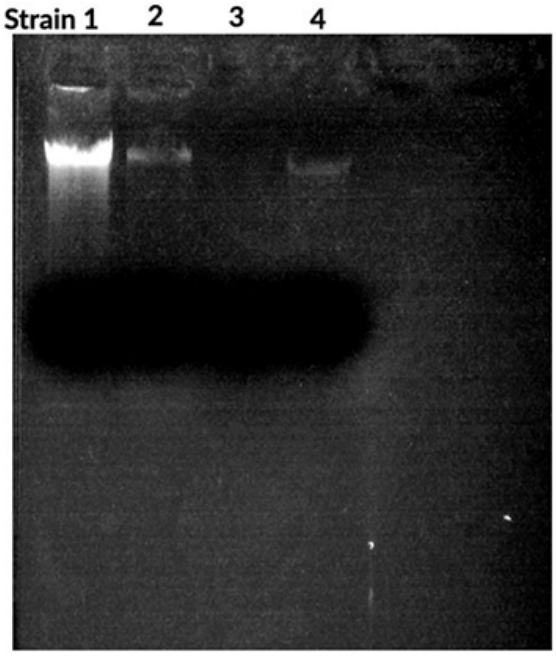

Fig. 2. Isolation of Plasmid DNA

In case of the plasmid DNA, strain 1 represented *Neisseria* sp from petrochemical effluent, stain 2 represented *Micrococcus* sp from petrochemical effluent, strain 3 represented *Pseudomonas* sp from textile effluent and strain 4 represented *Micrococcus* sp from textile effluent. This study primarily gave the information about the absence of plasmid DNA in *Pseudomonas* sp from textile effluent, so it might be concluded that this strain encodes the genes for biodegradation of phenol in genomic DNA.

Enzymes involved in the degradation of toluene, naphthalene, salicylate, octane etc. have been shown to be plasmid encoded (Barbly and Barbour, 1984; Nelson, 1990). Plasmids are also important in the development (transformation) of new organisms with enhanced degradative capability. Using molecular biology techniques, it is possible to slice pieces of DNA containing genes for specific degradative pathways into plasmids. These plasmids can then be introduced into a host organisms resulting in a recombinant or genetically engineered microorganisms (GEM) with new degradative capabilities (Brook and Madigan, 1991; Brand *et al.,* 1992). These are used to bioremediate contaminated sites mainly as organisms for bioaugumentation (Mc Clune *et al.,* 1989; Philips *et al.,* 1989; Focht, 1998). Appropriate environmental factors are essential for the performance of these organisms. A study by John and Okpokwasili (2015) has revealed a relationship between crude oil degradation and plasmid profile of nitrifying bacteria. It shows that nitrifying bacteria isolated from crude oil-contaminated ecosystem have ability to proliferate and degrade hydrocarbons. It can be deduced that their presence in polluted substrate encourage the development of adaptive features such as plasmid which support hydrocarbon co-metabolism. As a future perspective, the plasmid DNA can be removed from the strains and those without plasmid can be used to study the degradation potential of selected strains using the same effluent so that we can make sure that the degradation is plasmid mediated or not. Summers and Silver (1978) reported that in some cases the hydrocarbon degrading capabilities of some bacterial strains are plasmid borne. The plasmids that bear genes encoding for enzymes capable of degradation have been a great attraction. These plasmids, known as catabolic plasmids can give the organisms harbouring them the ability to degrade certain compounds. Sayer *et al.* (1990) reported the presence of catabolic plasmids in species of *Pseudomonas, Alcaligenes, Actinobacter, Flavobacterium, Klebsiella, Moraxella* and *Arthrobacter.*

Conclusion

As a result of the study, 10 potential phenol degrading bacteria were isolated from effluent treatment plants of petrochemical and textile industries. Growth and total phenol content were studied with varying concentrations of phenol (200ppm, 400ppm, 600ppm, 800ppm and 1000ppm). The selected strains showed maximum degradation potential in medium with 800ppm phenol. From the selected strains, again four strains were used for the isolation of genomic and plasmid DNA. Further future studies have to be conducted to investigate the plasmid mediated phenol degradation by the selected strains.

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