DOI : 10.35124/bca.2019.19.2.4635

Biochem. Cell. Arch. Vol. 19, No. 2, pp. 4635-4641, 2019 | www.connectjournals.com/bca | *ISSN 0972-5075*

LAB SCALE STUDY ON CHARACTERIZATION AND IDENTIFICATION OF PHENOL DEGRADING BACTERIA ISOLATED FROM PULP AND PAPER MILL EFFLUENT

Preeti Sachan[1*], Sangeeta Madan[1], Athar Hussain[2] and Utkarsh Singh[3]

[1]Department of Environmental Sciences, Kanya Gurukul Campus, Gurukul Kangri University, Haridwar - 249 404, India.
[2]Department of Civil Engineering, Ch. Brahm Prakash Government Engineering College, Jaffarpur, Delhi - 110 073, India.
[3]Department of Biotechnology, Baba Farid Institute of Technology, Dehradun - 248 007, India.
*e-mail: sachanpreeti84@gmail.com

(Received 13 April 2019, Revised 20 July 2019, Accepted 28 July 2019)

ABSTRACT : The untreated effluents from paper mills discharged into water bodies, damages the water quality and living organisms. Due to several limitations in the physicochemical treatment methods for treating industrial wastewaters the biological methods are more favourable alternative for the removal of pollutants. In the present study, phenol degrading activity by bacterial isolates collected from industrial effluent has been investigated. The isolated strains were further characterized by morphological, physiological, biochemical and 16S rRNA gene analysis. Isolates as *Staphylococcus sp.* (MH935803) and *Staphylococcus sciuri* (MH938044), has been identified in the present study. These two isolates have been identified as novel and potential aerobic bacterial strains that showed maximum survival in the phenol concentration up to 1600 mg/L and 1800 mg/L respectively. Further these strains may be used for biodegradation of phenol containing pulp and paper mill effluent. The study focuses on the characterization of efficient phenol degrading bacteria found in pulp and paper mill effluent which can be considered for use in large scale biorefining.

Key words : 16S rRNA, bioremediation, phenol, pulp paper mill effluent.

INTRODUCTION

In recent years, considerable attention has been paid to the industrial wastes, which are usually discharged on land or into different water bodies. As the industrial development is going on in India, the industrial activities are increasing and creating an adverse impact on agriculture as well as on living organisms. This is likely to result in the degradation of environment (Chhonkar *et al*, 2000). Various physico-chemical techniques have been studied for their applicability to the treatment of wastewater (Song *et al*, 2004; Badani *et al*, 2005; Rodrigues *et al*, 2007), but several limitations in the physicochemical methods make the biological methods a favourable alternative for the removal of pollutants in the industrial effluents. The process of biodegradation is a well-established and powerful technique for treating domestic and industrial effluents (Collern, 2003). Due to the modern trend towards enclosing process water systems, paper machine waters have become richer in nutrients for microbial growth. Suitable temperatures (30°–500°C) and a neutral pH favours the growth of bacteria.

A study conducted by Prabu and Udayasoorian (2005) white rot fungus was isolated from soil samples enriched by continuous paper mill effluent irrigation enabled identification of *Phanerochaete chrysosporium*. Organic and inorganic contents of the effluent also provide ample opportunity to the flourishing of a variety of pathogenic microorganism (Chandra *et al*, 2006). Due to high chemical diversity of the organic pollutants in paper mill effluent, a high variety of toxic effects on aquatic communities in the recipient water courses have been observed. These substances have been classified as carcinogenic, mutagenic, clastogenic and endocrinic (Karrasch *et al,* 2006). The untreated effluents from paper mills discharged into water bodies, damages the water quality and living organisms. Bioremediation is one of the most promising technologies that are expected to play an important role in waste site clean-up (Jeyasingh and Philip, 2005). Considering the above mentioned facts in view a study has been planned with the objective in order to study the isolation and characterization of the phenol degrading bacteria present in paper mill effluent in a laboratory scale.

MATERIALS AND METHODS

Growth media

Mineral salt medium (MSM) used for enrichment and screening of phenol degrading strains contained (mg/L): 400 K_2HPO_4, 200 KH_2PO_4, 400$(NH_4)_2SO_4$, 100NaCl, 100 $MgSO_4$, 10 $MnSO_4.H_2O_2$, 10 $Fe_2(SO_4)_3.H_2O$, 10 $Na_2MoO_4.2H_2O$. Phenol was used as sole carbon source for the present study.

Isolation and screening of phenol degrading bacterial strains

Effluent sample for the present study was collected in presterlized glass bottle from outlet of an Effluent Treatment Plant (ETP) of pulp and paper mill effluent industry. Effluent sample was filtered through ordinary filter paper in order to remove the suspended particles and solids.

For the isolation of pure cultures, Serial dilution technique has been carried out for the samples of paper mill effluent collected from effluent treatment plant. Pour plating was done from the dilutions of 10^{-6}, 10^{-7} and 10^{-8} in petriplates containing minimal salt medium (MSM) at 37°C. Single isolated colonies, which appeared on incubated plates were picked up with the help of sterile nichrome wire and streaked on fresh plates of the same medium. These plates were again incubated at 35±2°C for 24-48 hrs in incubator.

Phenol tolerant bacteria were screened from enrichment flask containing MSM with phenol as sole carbon source. The broth was taken with different concentration of phenol ranging from 0 mg/L to 2000 mg/L. The isolated strains were inoculated into medium with the help of inoculation loop and incubated on a shaking incubator (Biogen) at 125 rpm and 28°C±2°C for 48 hours in 500 mL conical flasks. Growth of bacterial cells at different phenol concentrations was determined by bacterial turbidity measurement at a wavelength of 610 nm after every 2 hours interval up to 48 hours incubation along with control by using UV-VIS Spectrophotometer (Systronic 118). The isolates were screened for further studies in order to assess the tolerance to phenol.

The selected isolates were purified by repeated streaking on MSM containing 100 mg/L of phenol, and the working culture was maintained by culturing in MSM containing 1000 mg/L of phenol at two weeks intervals (Ali *et al,* 1998).

Morphological and biochemical identification of bacterial isolates

For identification of strains the culture characteristics, morphological characteristics and biochemical tests were conducted and identified on the basis of characters as given in Bergy's manual of systematic bacteriology (Garrity, 2005).

By microscopic examination the selected isolates were studied morphologically (Gram's reaction, shape of colony, colony colour, margin, elevation spore formation, growth of colony, density and motility etc.). The isolates were identified and characterized on the basis of standard biochemical tests (Catalase Test, Phosph atase Test, amylase Test, Lipase Test, Gelatinase Test, Urease Test, Nitrate Reduction, Coagulases, Oxidase Test, Methyl Red Test and VP Test) (Cappuccino and Sherman, 1983).

Molecular Identification of bacterial isolates

Selected isolates SP-4 and SP-8 were identified by using 16Sr RNA gene analysis. For this, bacterial genomic DNA was extracted and purified by following the standard method (Baele *et al*, 2000) and evaluated on 1.2% agarose gel (Marmur, 1961).

For sequence analysis of ~1.5 kb 16S rRNA gene fragment, consensus primers were used and amplification performed by Taq DNA polymerase. The PCR product was bi-directionally sequenced using the universal forward and reverse primer set fD1 and rP2, respectively (Weisburg *et al*, 1991) using a thermocycler (Eppendorf). Reaction mixture contained reaction buffer (10X):5.0 µl, dNTPs mix (10 mM):5.0 µl, $MgCl_2$ (25mM):3.0 µl, Forward primer (10 pmol/µl):1.5 µl, Reverse primer (10 pmol/µl):1.5 µl, Template DNA: 5.0 µl, *Taq*DNA polymerase (5U/µl):0.5 µl, MQ water: 28.5 µl for final volume of 50 µl. PCR cycling parameters included an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute and extension at 75°C for 2 minutes and a final extension of 10 minutes at 75°C.

To confirm the amplicons the PCR products were separated on a 0.8% agarose low melting gel and the band corresponding to 1.5 kb 16S rRNA gene was purified using Qiagen Min Elute PCR purification kit and then quantified. Purified PCR products were sequenced by DNA sequencing service provided by Genei, Banglore following dideoxy chain-termination method.

Bioinformatics analysis and phylogenetic tree construction

A dendrogram was used to illustrate the relatedness of a sequence cluster produced in a phylogeny. The phylogenetic trees were constructed for about 10-18 most similar 16S rDNA gene sequences of established species. The 16S rDNA gene sequence has been used to carry out BLAST (http://www.ncbi.nlm.nih.gov/Blast.cgi)

(Altschul *et al*, 1997) with the nr (non-redundant) database of NCBI genbank database and RDP database, about 11 most similar established sequences were selected (Maidak *et al*, 2000). All the sequences were aligned using Clustal W. The evolutionary distance matrix based on Nucleotide Sequence Homology was generated using the Kimura-2 Parameter database and the phylogenetic tree was constructed in MEGA 6.0 software (Tamura *et al*, 2007) using Neighbor Joining method (Saitou and Nei, 1987).

RESULTS AND DISCUSSION

Isolation and screening of phenol tolerant bacterial isolates

Luxuriant growth has been observed in presence of bacterial isolates SP-4 and SP-8 with amended MSM in presence of 1% glucose (w/v), whereas no growth has been observed in absence of glucose. However rapid growth has been observed at phenol concentration of 0-1000 mg/L. According to growth studies pattern, SP-4 and SP-8 were able to tolerate a phenol concentration of 1600 mg/l and 1800 mg/L, respectively. No growth has been observed at phenol concentration of 2000 mg/L, as it may cause substrate inhibition (Yang and Lee, 2007).

Subsequent exposure to the increasing concentration of the phenolic compound from 0-2000 mg/L to isolated microorganisms can efficiently degrade phenol. Also it is a common technique used in the enrichment process. Rigo and Alegre (2004) conducted a study and concluded that isolated *Candida parapsilopsis* can effectively degrade phenol concentration of 1000 mg/L after screening.

Table 1(a) : Alignment view using combination of NCBI GenBank and RDP database for SP-4.

Alignment View	ID	Alignment results	Sequence description
	SP-4	0.96	Studied sample
	EU622581	0.97	*Staphylococcus sp.* PGBS005
	EU855191	0.99	*Staphylococcus sciuri* strain CTSP9
	GQ261178	0.97	*Staphylococcus sciuri* strain YL081101
	AB233330	0.94	*Staphylococcus fleurettii* strain GTC 1999
	DQ997837	1.00	*Staphylococcus aureus* strain ATCC 14458
	DQ127902	0.97	*Staphylococcus simiae* strain CCM 7229
	AY953148	0.95	*Staphylococcus croceolyticus*
	AM980864	0.99	*Staphylococcus aureus* strain ATCC 43300
	AF515587	0.98	*Staphylococcus xylosus*
	AB233330	0.94	*Staphylococcus fleurettii* strain GTC 1999

Table 1(b) : Alignment view using combination of NCBI GenBank and RDP database for SP-8.

Alignment View	ID	Alignment results	Sequence description
	SP-8	0.96	Studied sample
	EU855191	0.99	*Staphylococcus sciuri* St.CTSP9
	EU660348	0.98	*Staphylococcus sciuri* St. CM26
	AY820254	0.99	*Staphylococcus sciuri* St. SCBM1
	D83370	1.00	*Staphylococcus lentus* St. ATCC 29070
	NR_024670	1.00	*Staphylococcus vitulinus* St. ATCC 51145
	AB009942	1.00	*Staphylococcus pulvereri*
	AB233330	0.94	*Staphylococcus fleurettii* St. GTC 1999
	AB305019	1.00	*Staphylococcus aureus* St.SA1
	AY953148	0.95	*Staphylococcus croceolyticus*
	AF532917	1.00	*Staphylococcus pasteuri* strain

Morphological and biochemical characteristics of bacterial isolates

In the present study, two phenol degrading bacteria SP-4 and SP-8 were isolated from pulp and paper mill effluent. Both the bacterial isolates were found to be gram positive, cocci shaped, non-motile and non-spore forming bacteria. Both the isolates showed positive reactions in terms of catalase, phosphatase gelatinase, urease, nitrate reduction, oxidase, casinase, methyl red tests. However negative reactions have been observed for lipase, coagulase, voges-proskaeur tests by both the isolates. Also both the isolates ferment sugars (glucose, ribose, fructose, galactose, maltose, sorbitol, sucrose, dextrose, cellobiose, glycerol) and utilize amino acids (tyrosine, tryptophan, phenylalanine, cystine, lucine, proline, valine, threonine, histidine, alanine, isolucine, methionine,aspartic acid, arganine, serine, glutamic acid).

Molecular characterization based on 16 s rRNA gene analysis

The obtained results using morphological and biochemical identification technique has been assessed. From the present study it can be observed that the

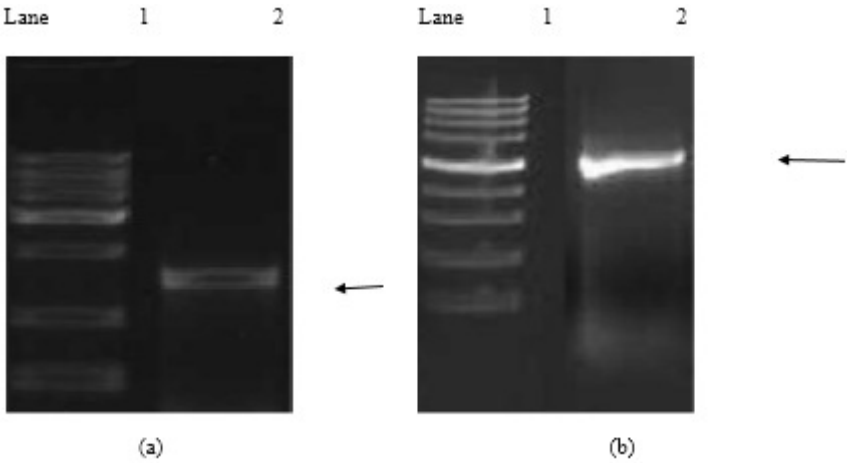

Fig. 1: (a) Gel Image of 16S r DNA amplicon of strain SP-4 **(b)** Gel Image of 16S r DNA amplicon of strain SP-8(Lane 1: DNA marker; Lane 2: 16S rDNA amplicon band)

AATACATGCAAGTCGAGCGAACAGATGAGAAGCTTGCTTCTCTGATGTTAGCGGCGG
ACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTCCGGGAAACC
GGGGCTAATACCGGATAATATTTTGAACCGCATGGTTCAATAGTGAAAGACGGTTTCG
GCTGTCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTAC
CAAGGCGACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGAC
ACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGC
CTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTGTT
AGGGAAGAACAAATTTGTTAGTAACTGAACAAGTCTTGACGGTACCTAACCAGAAAG
CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTATGTGGCAAGCGTTATCCGG
AATTATTGGGCGTAAAGCGCGCGTACGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCG
GCTCAACCGGGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTG
GAATTCCATGTGTAGCGGTGAAATGCGCAGAGATGTGGAGGAACACCAGTGGCGAAG
GCGGCTCTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCC
GCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAG
GTTGGAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAA
TTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACCGCTCTAGAGAT
AGAGTCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGT
GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCAT
CATTAAGTTGGGCACTCTAGGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT
GACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGATAAT
ACAAAGGGCAGCGAATCCGCGAGGCCAAGCAAATCCCATAAAATTATTCTCAGTTCG
GATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGC
ATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGT
TTGTAACACCCGAAGCCGGTGGAGTAACCTTTTAGGAGCTAGCCGTCGAAGGTGGGA
CAAATGATTGGGGT

Fig. 2 (a) : Aligned sequenced data of 16 S r RNA gene of SP-4 (1452 bp).

morphological and biochemical identification technique is very well supported by molecular identification techniques. Similar study conducted by Hagstrom *et al* (2000) reported that 16S r RNA sequence having similarity of ≥ 97% is a authentic level to match bacteria into species.

Result obtained on fragment of 16S rRNA gene has been amplified by PCR and resolved on agarose gel where a single discrete PCR amplicon band of 1500 bp has been observed (Fig. 1). Using universal primers, 1452bp of SP-4 and 1468 bp of SP-8 rRNA genes were amplified, sequenced and submitted to gene bank (Fig. 2). Also the accession numbers MH 935803 and MH938044 were obtained for SP-4 and SP-8 respectively. Nucleotide blast searches for the amplified 16S r RNA gene sequence of SP-4 showed 97% sequence similarity with *Staphylococcus sp.* PGBS005 (EU622581) (Table 1). Whereas, SP-8 showed 98% sequenced similarity with *Staphylococcus sciuri* strain CM26 (EU660348). Therefore, SP-4 could be characterized as *Staphylococcus* sp. and designated as *Staphylococcus* sp. strain SP-4 (MH935803). Similarly, SP-8 could be characterized as *Staphylococcus sciuri* and was designated as *Staphylococcus sciuri* strain SP-8 (MH938044).

Besides analyzing bacteria with morphological and biochemical methods, microbiologist rely on molecular tools particularly rRNA gene sequencing. The direct amplification of 16S r RNA gene enables one to identify the exact sample taken from environment (Olsen *et al*, 1986).

Phylogenetic analysis of 16 S rRNA gene sequence

In order to determine the taxonomic position of strains SP-4 and SP-8 among other strains of the genus Staphylococcus, the evolutionary distance matrix was

TGCCTAATACATGCATCTCGAGCGAACAGATGAGAAGCTTGCTTCTCTGATGTTAGCG
GCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTCCGGGA
AACCGGGGCTAATACCGGATAATATTTTGAACCGCATGGTTCAATAGTGAAAGACGG
TTTCGGCTGTCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGG
CTTACCAAGGCGACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACT
GAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCG
AAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCT
GTTGTTAGGGAAGAACAAATTTGTTAGTAACTGAACAAGTCTTGACGGTACCTAACCA
GAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTT
ATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAG
CCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGG
AGAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTG
GCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAA
ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGG
GGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGA
CCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACCGCTC
TAGAGATAGAGTCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAATTCCCGCAACGAGCGCAACTCTTATGCTTAT
TTGCCATCATTAAGTTGGGCACTCTAGGTTGACTGCCGGTGACAAACCGGAGGAAGGT
GGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATG
GATAATACAAAGGGCAGCGAATCCGCGAGGCCAAGCAAATCCCATAAAATTATTCTC
AGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAG
ATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCAC
GAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCTTTTAGGAGCTAGCCGTCGAAG
GTGGGACAAATGATGGTGTCCTCTTTTCCCG

Fig. 2(b) : Aligned sequenced data of 16 S r RNA gene of SP-8 (1468 bp).

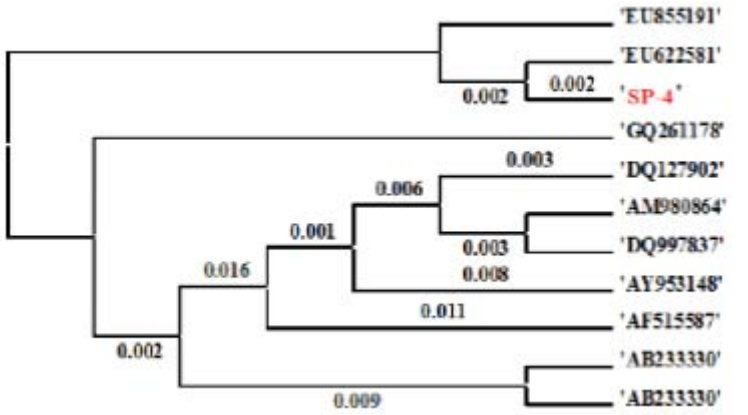

Fig. 3(a) : Phylogenetic Tree in MEGA 6.0 software using Neighbor Joining method for SP-4.

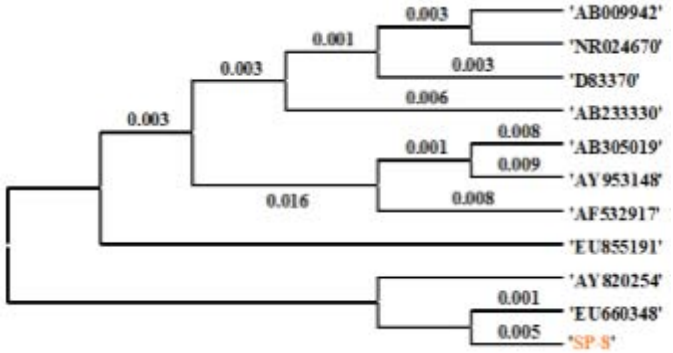

Fig. 3(b): Phylogenetic Tree in MEGA 6.0 software using Neighbor Joining method for SP-8.

generated by using Kimura-2 software (Table 2). Also, the phylogenetic tree was constructed using Mega 6.0 software (Fig. 3). The data summarized in Table-2 indicates the nucleotide similarity (above diagonal) and distance (below diagonal) identities between the identified sample SP-4 and SP-8 with ten other closest homologs microbes. The evolutionary distance dendograms revealed that all the sequences obtained from the database related to strains SP-4 and SP-8 fell into class Bacilli and domain bacteria.

Previously, it has been observed that members of class bacilli possess the ability of only degrading the aromatic hydrocarbons (Abd-El-Haleem *et al*, 2002). Whitely and Bailey (2002) have also demonstrated that organisms are dominant prevalent in industrial phenolic waste system by using molecular characterization and phylogenetic tree. The phenol degrading isolates obtained

Table 2(a) : Distance Matrix based on Nucleotide Sequence Homology (Using Kimura-2 Parameter for SP-4).

Distance Matrix												
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
GQ261178	1	—	0.973	0.999	0.973	0.989	0.972	0.997	0.970	0.989	0.972	0.995
DQ127902	2	0.027	—	0.974	0.985	0.964	0.994	0.971	0.979	0.964	0.994	0.969
EU855191	3	0.001	0.026	—	0.974	0.989	0.972	0.998	0.970	0.989	0.972	0.996
AY953148	4	0.027	0.015	0.026	—	0.964	0.983	0.971	0.981	0.964	0.983	0.969
AB233330	5	0.011	0.036	0.011	0.036	—	0.966	0.987	0.966	1	0.966	0.984
AM980864	6	0.029	0.006	0.028	0.017	0.034	—	0.970	0.979	0.966	1	0.968
EU622581	7	0.003	0.029	0.002	0.029	0.013	0.030	—	0.968	0.987	0.970	0.998
AF515587	8	0.030	0.021	0.030	0.019	0.034	0.021	0.032	—	0.966	0.979	0.966
AB233330	9	0.011	0.036	0.011	0.036	0.000	0.034	0.013	0.034	—	0.966	0.984
DQ997837	10	0.029	0.006	0.028	0.017	0.034	0.000	0.030	0.021	0.034	—	0.968
SP-4	11	0.005	0.031	0.004	0.031	0.016	0.032	0.002	0.035	0.016	0.032	—

Table 2(b) : Distance Matrix based on Nucleotide Sequence Homology (Using Kimura-2 Parameter for SP-8).

Distance Matrix												
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
AY820254	1	—	0.990	0.999	0.989	0.991	0.972	1	0.974	0.973	0.990	0.995
AB009942	2	0.010	—	0.990	0.991	0.994	0.969	0.990	0.968	0.972	1	0.985
EU660348	3	0.001	0.011	—	0.988	0.990	0.972	0.999	0.973	0.972	0.990	0.994
AB233330	4	0.011	0.009	0.012	—	0.989	0.966	0.989	0.964	0.967	0.991	0.984
D83370	5	0.009	0.006	0.010	0.011	—	0.969	0.991	0.968	0.972	0.994	0.986
AB305019	6	0.028	0.031	0.028	0.034	0.031	—	0.972	0.983	0.984	0.969	0.967
EU855191	7	0.000	0.010	0.001	0.011	0.009	0.028	—	0.974	0.973	0.990	0.995
AY953148	8	0.026	0.032	0.027	0.036	0.032	0.017	0.026	—	0.982	0.968	0.969
AF532917	9	0.027	0.029	0.028	0.034	0.029	0.016	0.027	0.018	—	0.972	0.968
NR_024670	10	0.010	0.000	0.011	0.009	0.006	0.031	0.010	0.032	0.029	—	0.985
SP-8	11	0.005	0.015	0.006	0.016	0.014	0.033	0.005	0.031	0.032	0.015	—

in present study may be utilized in future work to develop good and efficient treatment systems such as microbial consortia which can be used for industry. Isolation and characterization may provide a good initiative for the discovery of such phenol degrading beneficial enzymes.

CONCLUSION

From the present study, it can be inferred that isolation, screening and identification methods are efficient methods to identify phenol degrading bacteria from various efficient samples. As per the obtained growth studies pattern, SP-4 and SP-8 were able to tolerate a phenol concentration of 1600 mg/l and 1800 mg/L respectively. No growth has been observed at phenol concentration of 2000 mg/L, as it may cause substrate inhibition. The evolutionary distance dendograms revealed that all the sequences obtained from the database related to strains SP-4 and SP-8 fell into class Bacilli and domain bacteria. From the environmental and economical point of view naturally exploring of phenol degrading bacteria in the environment is important in the field of biological treatment of industrial effluents. This will help to overcome costly hurdles in biological treatment process.

REFERENCES

Abd-El-Haleem D, Moawad H, Zaki E A and Zaki S (2002) Molecular characterization of phenol-degrading bacteria isolated from different Egyptian ecosystems. *Microb Eco*. **43**, 217-224.

Ali S, Fernandez-Lafuente R and Cowan D A (1998) Meta-pathway degradation of phenolics by thermophilic *Bacilli*. *Enz Micro Technol*. **23**, 462-468.

Altschul S F, Madden T L, Scaffer A A, Zhang J, Zhang Z, Miller W and Lipman D J (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search program. *Nuc Acids Res*. **25**, 3389-3402.

Badani Z, Ait-Amar H, Si-Salah A and Fuchs W (2005) Treatment of textile wastewater by membrane bioreactor and reuse. *Desalinat* **185**, 411-417.

Cappuccino J C and Sherman N (1992) *Microbiology: A Laboratory Manual*. Benjamin/cummings Pub Co, New York **3**, 125-179.

Chandra R, Singh S and Raj A (2006) Seasonal bacteriological analysis of Gola river water contaminated with pulp paper mill waste in Uttaranchal, India. *Environ Monit Assess* **118**, 393-406.

Chhonkar P K, Datta S P, Joshi H C and Pathak H (2000) Impact of industrial effluents on soil health and agriculture Indian experience: Part-II – Tannery and textile industrial effluents. *J Sci Ind Res*. **59**, 446-454.

Collern E (2003) Methods in Biotechnology. In *Bioremediation*

protocols, ed. D. Sheehan, Humana Press Inc., Totowa, NJ.

Garrity G (2005) The Proteobacteria, Part B: The Gammaproteobacteria. In : *Bergey's manual of Systematic bacteriology*, ed. Brenner D J, Kreig N R, Staley J T. Springer, New York, 2: 607-624.

Hagstrom A, Pinhassi J and Zweifel U L (2000) Biogeographical diversity among marine bacterioplankton. *Aquat. Microbiol. Ecol.* **21**, 231-244.

Jeyasingh J and Philip L (2005) Bioremediation of chromium contaminated soil: optimization of operating parameters under laboratory conditions. *J Hazard Mater* **118**, 113-120.

Karrasch B, Parra O, Cid H, Mehrens M, Pacheco P, Urrutia R, Valdovinos C and Zaror C (2006) Effects of pulp and paper mill effuents on the microplankton and microbial self-purification capabilities of the Biobio River, Chile. *Sci Total Environ.* **359**, 194-208.

Maidak B L, Cole J R, Lilburn T G, Parker C T, Saxman P R, Stredwick J M, Garrity G M, Li B, Olsen G J, Pramanik S, Schmidt T M and Tiedje J M (2000) The RDP (Ribosomal database Project) continues, *Nuc Acids Res*. **28**, 173-174.

Marmur J (1961) A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol*. **3**, 208-218.

Prabu P C and Udayasoorian C U (2005) Biodecolorization of phenolic paper mill effluent by ligninolytic fungus, *Trametes versicolor*. *J Biol Sci*. **5**, 558-561.

Rigo M and Alegre R M (2004) Isolation and selection of phenol-degrading microorganisms from industrial wastewaters and kinetics of the biodegradation. *Folia Microbiol.* **49**, 41-45.

Rodrigues A, Xavier S and Bernardes F (2008) Application of photo electrochemical-electro-dialysis treatment for the recovery and reuse of water from tannery effluents. *J Clean Prod*. **10**, 1-7.

Saitou N and Nei M (1987) The Neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*. **4**, 406-425.

Song Z, Williams C L and Edyvean R G J (2004) Treatment of tannery wastewater by chemical coagulation. *Desalinat* **164**, 249-259.

Tamura K, Dudley J, Nei M and Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol*. **16**, 111-120.

Weisburg W G, Barns S M, Pelletier D A and Lane D J (1991) 16S Ribosomal DNA amplification for phylogenetic study. *J Bacteriol*. **173**, 697-703.

Whiteley A S and Bailey M J (2000) Bacterial community structure and physiological state within an industrial phenol bioremediation system, *Appl Environ Microbiol*. **66**, 2400-2407.

Yang C F and Lee C M (2007) Enrichment, isolation, and characterization of phenol degrading *Pseudomonas resinovorans* strain P-1 and *Brevibacillus* sp. strain P-6. *Int Biodeter Biodeg*. **59**, 206-210.