Pak. J. Agri. Sci., Vol. 52(1), 219-231; 2015
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
http://www.pakjas.com.pk

CHARACTERIZATION AND IDENTIFICATION OF PHENOL DEGRADING BACTERIA ISOLATED FROM INDUSTRIAL WASTE

Nazir Ahmad[1,2], Iftikhar Ahmed[1,2,*], Muhammad Iqbal[1,2], Nauman Khalid[3], Farrakh Mehboob[1,4], Karam Ahad[4] and Ghulam Muhammad Ali[1,2]

[1]PARC Institute of Advanced Studies in Agriculture (PIASA), National Agricultural Research Centre (NARC), Park Road, Islamabad-45500, Pakistan; [2]National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Centre (NARC), Park Road, Islamabad-45500, Pakistan; [3]Graduate School of Agricultural and Life Sciences, The University of Tokyo, 11-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan; [4]Ecotoxicology Research Institute, National Agricultural Research Centre (NARC), Islamabad, Pakistan.
[*]Corresponding author's e-mail: iftikharnarc@hotmail.com

Phenol is a toxic organic pollutant to living cells and its biodegradation is considered the best method due to its environment friendly nature and cost effectiveness. In this study, eight bacterial strains were isolated through enrichment on mineral salt media supplemented with 300 mgL^{-1} phenol. The isolated strains were identified by 16S rRNA gene sequence analysis and belonged to genera: *Rhodococcus, Stenotrophomonas, Lysinibacillus, Comamonas, Microbacterium, Pseudomonas* and *Halomonas*. The results of phenol biodegradation experiments (conducted at pH 7 and 30°C temperature) showed that the strains could degrade 750 mg L^{-1} phenol within 40 to 96 hours. The average phenol degradation rate by the strains was 12.5 to 34.8 $mgL^{-1}h^{-1}$. The most rapid phenol degradation was observed for *Rhodococcus* sp. NCCP-309 and *Rhodococcus* sp. NCCP-312, whereas, *Stenotrophomonas* sp. NCCP-311, *Lysinibacillus* sp. NCCP-313, *Comamonas* sp. NCCP-314 and *Microbacterium* sp. NCCP-351 took longer time in phenol degradation. The results of our study suggested that these strains are efficient in phenol biodegradation and can be used for the bioremediation of waste water containing phenol.
Keywords: Phenol biodegradation, Industrial waste, Organic pollution, *Rhodococcus, Stenotrophomonas, Lysinibacillus*

INTRODUCTION

Phenol is an aromatic hydrocarbon with good solubility in aqueous media. Among the organic pollutants, phenol is of special importance because of its widespread distribution (Sandhu *et al.,* 2009) and toxicity to humans, plants and marine life even at low concentration (Das and Santra, 2012). Phenol is used in many industries including petrochemicals and oil refining (Movahedyan *et al.,* 2009), pharmaceuticals, plastics, resin manufacturing and coke plants (Cao *et al*., 2011) for manufacturing different products and is released into the environment (water) through discharge of waste.

On the basis of toxicity and solubility in water, phenol is classified as a priority pollutant by the U.S Environmental Protection Agency (Tsao *et al*., 1998). Water is contaminated with phenol mainly from the industrial waste and concentrations of more than 50 mg L^{-1} has been found in waste (Agency for Toxic Substances and Disease Registry (ATSDR), 2011). A phenol concentration of 0.5 mg L^{-1} in industrial waste is permitted by the Environmental Protection Agency (Giti *et al.,* 2005). The European Community (EC) directive for drinking water officially permitted an acceptance level of 0.5 μg L^{-1} phenol in water for human consumption (Steiner *et al.,* 2008).

Because of toxicity of phenol, deleterious changes occur in ecosystem if it is liberated without further processing (Pradeep *et al.,* 2011). Phenol is detrimental for a number of organisms including humans (Ehrt *et al*., 1995). Many disorders of central nervous system, hypothermia, skin damage, whitening of the cornea and sometimes complete blindness, hepatic damage etc. have been reported in humans due to phenol toxicity (Naresh *et al*., 2012). It is reported that 1-10 mM phenol causes break down in vegetation growth, nutrient intake, and transpiration in plant (Alber *et al.,* 1989). In experimental animals, phenol is reported harmful to skeleton, nephrotoxic, immuno-toxic and feto-toxic. Phenol causes change in the DNA sequence, chromosomal abnormality and impulsive synthesis of DNA in model animals (Brown, 2008). Phenol is lethal at a concentration of 5-25 mg L^{-1} for fish (Nuhoglu and Yalcin, 2005). Contamination of phenolic compounds in the food chain is a big issue and its elimination to meet the environment regulation is very necessary (US-EPA, 1979).

Phenol can be remediated from environment by several physical and chemical methods, such as solvent extraction (Lazarova and Boyadzhieva, 2004), adsorption (Carmona *et al*., 2006), chemical oxidation and incineration (Wu *et al*., 2000). But these methods are not environment friendly because of the production of toxic secondary intermediates, high cost and health hazards (Yan *et al.,* 2006; Bai *et al*.,

2007; Zhai *et al.,* 2012). Biodegradation is the best way to cope with the problem since this process is cheap, environment friendly and easy to handle (Basha *et al.,* 2010). Bacteria oxidize phenol into CO_2 and H_2O during metabolic processes (Loh and Chua, 2002).

Phenol is widespread in distribution (Park *et al.,* 2012) and many microorganisms can utilize phenol for their growth as primary energy source (Tuah *et al.,* 2009). Such microorganisms have potential to degrade phenol (Nair *et al*., 2008). Several phenol degrading bacteria have been isolated like *Gulosibacter* sp. (Zhai *et al.,* 2012), *Acinetobacter* sp. (Ahmad *et al.,* 2011), *Bacillus cereus* (Banerjee and Ghoshal, 2010), *Staphylococcus epidermis* (Mohite *et al*., 2010), *Xanthobacter flavu*s (Lowry *et al.,* 2009), *Burkholderia cepacia* (El-Sayed *et al.,* 2003), *Pseudomonas* (Mahiudddin *et al.,* 2012; Ahmad *et al.,* 2014) and *Rhodococcus* (Rehfuss and Urban, 2005), etc.

The objectives of current study were to isolate and characterize indigenous bacterial strains from industrial waste that can tolerate toxic concentrations of phenol and to further test their phenol degrading ability.

MATERIALS AND METHODS

***Isolation and purification of the strains*:** All the chemicals of reagent grade were purchased from Merck (Darmstadt, Germany) and Sigma Chemicals Co. (St. Louis, MO, USA). Phenol having purity of 99.0% was used in the experiments. Mineral salt medium (MSM, Sigma, USA) was used for determination of phenol tolerance and degradation. This medium composed of K_2HPO_4 (0.4 g L^{-1}), KH_2PO_4 (0.2 g L^{-1}), NaCl (0.1 4 g L^{-1}), $MgSO_4$ (0.1 4 g L^{-1}), $(NH_4)_2SO_4$ (0.4 g L^{-1}) and 0.01 g L^{-1} of $MnSO_4.H_2O$, $Fe_2(SO_4).3H_2O$ and $Na_2MoO_4.2H_2O$. For isolation of phenol degrading bacteria, samples of sludge were collected from Bioremediation Site-1, National Agricultural Research Centre, Islamabad, Pakistan and from a combined drainage of an Industrial area in Sector I-9, Islamabad, Pakistan. Sample (10 mL) were added to 90 mL MSM supplemented with 300 mg L^{-1} phenol as sole source of carbon and energy to enrichment of phenol tolerant bacteria. The pH of medium was adjusted to 7. The flasks were incubated at 28°C for 3 days on a rotary shaker (120 rpm). After 3 days of enrichment, 2-3 drops of the sample were spread on MSM agar plates containing 300 mg L^{-1} phenol. The plates were incubated at 28°C. The individual colonies were sub-cultured on same medium to get pure culture of single strain. The isolated strains were differentiated morphologically on the basis of colony shape, color, surface, margins, elevation and opacity. The purified culture of strains was stocked in glycerol (30% w/v) at –20°C for further experiments.

***Identification and phylogenetic analysis of isolated strains*:** The isolated strains were identified using 16S rRNA gene sequence, which was amplified by colony PCR method described by Ahmed *et al.* (2007). The PCR reaction was performed in a thermal cycler (Applied Biosystems, Veriti 96-wells, U.S.A) by mixing of template DNA with TAKARA Pre-mix Ex-Taq (25 µL), universal forward and reverse primers (10 pmol μL^{-1}): 2 µL of 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 2 µL of 1510R (5′-GGCTACCTTGTTACGA-3′) in a total volume of 50 µL. The PCR cycling parameters consisted of an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1.5 min and a final extension for 7 min at 72°C. An amplicon of approximately 1.5 kb of 16S rRNA gene was purified using purification kit (Invitrogen) according to manufacturer's protocol and was sequenced using universal forward 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse 1492R (5'-ACCTTGTTACGACTT-3') primers from Macrogen, Korea (http://dna.macrogen.com/en). The sequence obtained were assembled using BioEdit software to get the consensus sequence for each strain. The sequences of all the stains were submitted to DNA Data Bank of Japan (DDBJ) under the accession numbers as mentioned in Table 1. The strains were identified using 16S rRNA gene sequence on Ez-Taxon Server (http://eztaxon-e.ezbiocloud.net) and BLAST search on DDBJ / NCBI servers. Sequences of closely related validly named type strains were selected and retrieved from the database of EzTaxon Server for constructing phylogenetic tree. The alignment and editing were performed using CLUSTAL X version 1.8msw; (Thompson *et al.,* 1994) and BioEdit (Hall, 1999) packages. Ambiguous positions and gaps were excluded during calculations. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura *et al*., 2011) in Neighbor joining algorithm. The stability of the relationship was assessed by bootstrap analysis,(Felsenstein, 2005) by performing 1,000 re-sampling for the tree topology of the neighbour-joining data.

***Biochemical characterization of the isolated strains*:** The isolated strains were characterized for the utilization of various organic and inorganic compounds. For this purpose, 16-18 h of pure bacterial culture was used to inoculate the API 20E kit (bioMerieux, France) according to the manufacturer's protocol and incubated at 28°C for 24-48 h before reading the results.

***Phenol toxicity tolerance and biodegradation*:** The strains were grown in MSM broth supplemented with 200 mg L^{-1} phenol in sterilized test tubes at 28°C for 2-3 days on shaker. This primary culture of each strain was used to inoculate the MSM broth containing various phenol concentrations (0, 250, 500, 750 and 1000 mg L^{-1}). Growth of each strain was determined by taking optical density (OD) using a spectrophotometer (IMPLEN, Germany) at 600 nm wavelength at different time intervals depending upon the growth pattern of strains.

Table 1. Identification of isolated strains based on 16S rRNA gene sequence and their accession numbers published in DNA database.

Strain ID	Strain Name / Genus	No. of nucleotides of 16S rRNA gene	Accession number of 16S rRNA gene	Closely related taxa identified by using the EzTaxaon Server Database (http://eztaxon-e.ezbiocloud.net)	Sequence similarity (%) of 16S rRNA gene with closely related taxa	Sequence query coverage (%)	No. strains with >97% sequence similarity
NCCP-309	*Rhodococcus* sp.	1416	AB734810	*Rhodococcus phenolicus* (AM933579)	98.94	98.0	9
NCCP-311	*Stenotrophomonas* sp.	1416	AB734811	*Stenotrophomonas geniculata* (AB021404)	99.64	96.3	13
NCCP-312	*Rhodococcus* sp.	1394	AB734812	*Rhodococcus zopfii* (AF191343)	99.93	96.5	7
NCCP-313	*Lysinibacillus* sp.	788	AB734813	*Lysinibacillus sphaericus* (CP000817)	99.87	53.5	5
NCCP-314	*Comamonas* sp.	1402	AB734814	*Comamonas thiooxidans* (DQ322069)	100.0	96.4	3
NCCP-351	*Microbacterium* sp.	930	AB734815	*Microbacterium paraoxydans* (AJ491806)	99.25	64.3	>33
NCCP-407	*Pseudomonas* sp.	1126	AB734816	*Pseudomonas plecoglossicida* (AB009457)	99.38	77.1	30
NCCP-178	*Halomonas* sp.	1417	AB698809	*Halomonas elongata* (FN869568)	99.72	97.1	8

Table 2. Biochemical characteristics of *Rhodococcus* sp. NCCP-309, *Rhodococcus* sp. NCCP-312, and *Stenotrophomonas* sp. NCCP-311, *Comamonas* sp. NCCP-314 and *Microbacterium* sp. NCCP-351

Biochemical tests	*Rhodococcus* sp. NCCP-309	*Rhodococcus* sp. NCCP-312	*Stenotrophomonas* sp. NCCP-311	*Comamonas* sp. NCCP-314	*Lysinibacillus* sp. NCCP-313	*Microbacterium* sp. NCCP-351
β-galactosidase	+	.	+	–	–	–
Arginine dihydrolase	–	–	+	–	–	–
Lysine decarboxylase	–	–	+	–	–	–
Ornithine dacarboxylase	–	–	–	–	–	–
Citrate utilization	–	–	+	–	–	–
H_2S production	–	–	–	–	–	–
Urease	–	–	–	–	–	–
Tryptophane deaminase	–	–	–	–	–	–
Indole production	–	–	–	–	–	–
Voges-Proskauer reaction	+	–	–	–	–	–
Hydrolysis of gelatin	–	–	+	–	+	–
Fermentation/Oxidation of:						
Glucose	–	–	–	–	–	–
Mannitol	–	–	–	–	–	–
Inositol	–	–	–	–	–	–
Sorbitol	–	–	–	–	–	–
Rhamnose	–	–	–	–	–	–
Sacharose	–	–	–	–	–	–
Melibiose	–	–	–	–	–	–
Amygdalin	–	–	–	–	–	–
Arabinose	–	–	–	–	–	–
NO_3 reduction	+	–	+	+	+	+

Promising strains were selected on the basis of their maximum tolerance level to phenol for biodegradation studies. These selected strains were inoculated in MSM broth having 750 mg L^{-1} phenol for 2-3 days at 28°C. The culture sample was collected at specific intervals depending upon growth patterns of the strain. An aliquot of each culture (1 mL) was collected at different time intervals and optical density (OD_{600}) was determined at 600 nm on spectrophotometer. The cells were removed by centrifugation (13000 rpm, 5 min) and phenol was quantified in the supernatant by High Performance Liquid Chromatography (HPLC). The HPLC analysis was carried out on Interface 900 Series HPLC System (Perkin Elmer, U.S.A) equipped with binary LC Pump 250, LC Oven 101, Column C18, and LC 295 UV/Vis detector. Mobile phase included acetonitrile and water (60:40) with a flow rate of 0.8 mL min^{-1} and detector was set at a wave length of 280 nm. Identification of phenol peak was done on the basis of retention time and quantification on the basis of six point external standard calibration curve.

RESULTS

***Isolation and identification of the isolated strains*:** Phenol degrading strains isolated from the samples collected from Bioremediation Site-1, NARC and Industrial Area I-9, Islamabad grew well on MSM medium enriched initially with 300 mg L^{-1} phenol. Preliminary characterization was done based on strain morphology (viz., colony morphology, color, margins, surface, elevation and opacity). Based on colony morphology, eight different strains were observed that were designated as NCCP-309, NCCP-311, NCCP-312, NCCP-313, NCCP-314, NCCP-351, NCCP-407 and NCCP-178. The colonies of all isolated strains were circular in shape except NCCP-314, which produced irregular colonies. The colony margins in most of these strains were entire except NCCP-313 (undulate) and NCCP-178 (lobate). Similarly, majority of the isolated strains were opaque except NCCP-311 and NCCP-407 (transparent). Color variation was observed in most of these strains.

Based on sequences of 16S rRNA genes, the isolated strains NCCP-309, NCCP-311, NCCP-312, NCCP-313, NCCP-314, NCCP-351, NCCP-407 and NCCP-178 showed similarity to *Rhodococcus phenolicus* (98.9%), *Stenotrophomonas geniculata* (99.6%), *Rhodococcus zopfii* (99.9%), *Lysinibacillus sphaericus* (99.9%), *Comamonas thiooxidans* (100%), *Microbacterium paraoxydans* (99.2%), *Pseudomonas plecoglossicida* (99.4%) and *Halomonas elongate* (99.7%), respectively (Table 1). The resulting phylogenetic tree depicts the phylogenetic relationship of the selected strains to closely related validly named species (Fig. 1–5). Among these strains, *Rhodococcus* sp. NCCP-309, *Rhodococcus* sp. NCCP-312, *Stenotrophomonas* sp. NCCP-311, *Lysinibacillus* sp. NCCP-313, *Comamonas* sp. NCCP-314 and *Microbacterium* sp. NCCP-351 were selected for further evaluation of their phenol degrading abilities.

***Biochemical characterization of selected isolated strains*:** Table 2 shows biochemical characterization of selected strains. After 48 h of incubation, the strain *Rhodococcus* sp. NCCP-309 showed positive results for *β*-galactosidase, sodium pyruvate and reduction of nitrite to N_2 and negative for the rest of substrates tested. Similarly, the strain *Rhodococcus* sp. NCCP-312 showed negative results for the entire substrates tested. However, *Stenotrophomonas* sp. NCCP-311 showed positive results for *β*-galactosidase, arginine dihydrolase, lysine decarboxylase, citrate utilization, sodium pyruvate, gelatinase and NO_2 production and negative for the rest of substrates tested. In comparison, *Comamonas* sp. NCCP-314 showed positive results only for NO_2 production and negative for the rest of substrates. *Lysinibacillus* sp. NCCP-313 showed positive results for gelatinase and reduction to N_2 gas and negative for all substrates tested. Similarly, *Microbacterium* sp. NCCP-351 showed positive result only for reduction to N_2.

***Phenol tolerance of the isolated strains*:** The selected strains were grown in MSM supplemented with different concentration of phenol (0, 250, 500, 750 and 1000 mg L^{-1}) as a sole source of carbon and energy at 28°C. No growth of any strain was observed in the absence of phenol, while significant growth was observed with the rest of concentrations. *Rhodococcus* NCCP-309 was incubated at 28°C for a total period of 118 h. At 250 and 500 mg L^{-1}, no lag phase was observed. Log phase was started from the time of inoculation and continued up to 24 and 51 h with maximum optical densities (OD) of 0.6 and 0.91, respectively, at 600 nm. At 750 mg L^{-1}, a lag phase of 24 h was observed and log phase was started after 24 h of inoculation and continued up to 51 h. Maximum growth was observed at 51 h of inoculation with OD of 1.17. At 1000 mg L^{-1} concentration, a longer lag phase was observed that started from the time of inoculation and continued up to 51 h, then log phase was started and continued up to 75 h. Maximum growth was observed at 75 h of inoculation and the OD value was 0.74 at 600 nm which was higher than that of 250 mg L^{-1} and lower than 750 and 500 mg L^{-1} (Fig. 6a).

Stenotrophomonas sp. NCCP-311 was incubated at 28°C for a total period of 66 h. At 250 mg L^{-1} dose, lag phase was started from the time of inoculation and continued up to 18 h. Log phase was started after 18 h of inoculation and continued up to 43 h. Maximum OD of 0.59 showing the highest growth was observed at 43 h. Similarly, at 500 and 750 mg L^{-1} phenol concentration, no lag phase was present. Maximum growth was observed at 2 days with OD of 0.45 and 0.93 at 600 nm. At 1000 mg L^{-1} phenol concentration, no growth was observed (Fig 6b).

Similarly, *Rhodococcus* NCCP-312 was incubated for a total period of 53 h. At 250, 500 and 750 mg L^{-1}, no lag phase was observed and log phase started from the time of inoculation

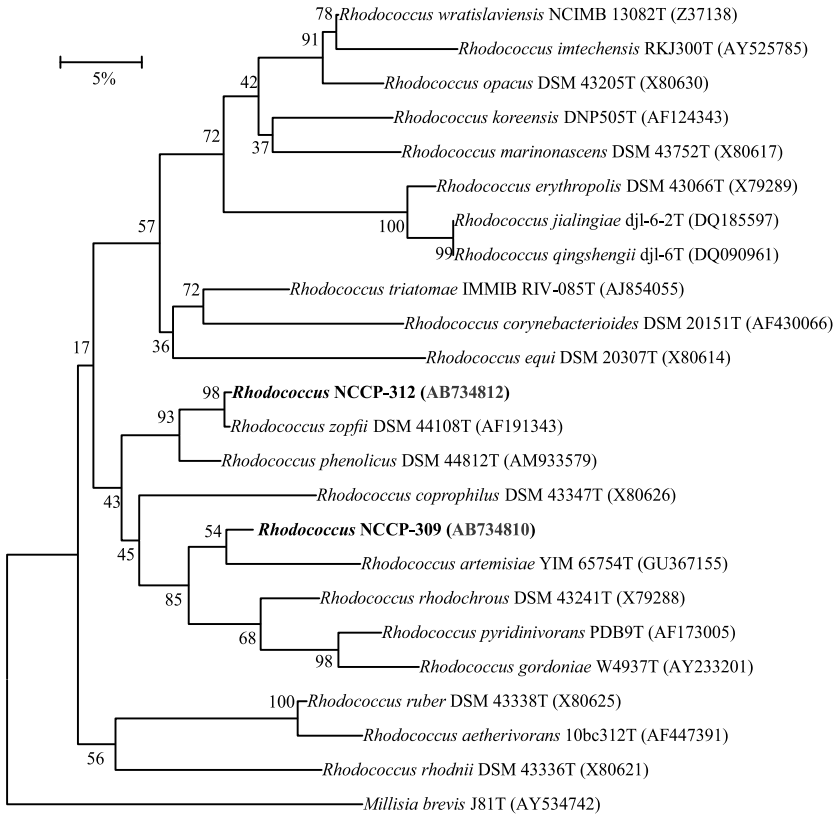

Figure 1. Phylogenetic tree showing the inter-relationships of two strains (*Rhodococcus* sp. NCCP-309 and *Rhodococcus* sp. NCCP-312) with the most closely related species inferred from sequences of 16S rRNA gene. *Millisia brevis* (AY534742) was used as an out group. The tree was generated using the Neighbour-Joining method. Bootstrap values expressed as a percentage of 1000 replications, are given at the branching point. The Bar shows 5% sequence divergence. The accession number of each strain is shown in parenthesis.

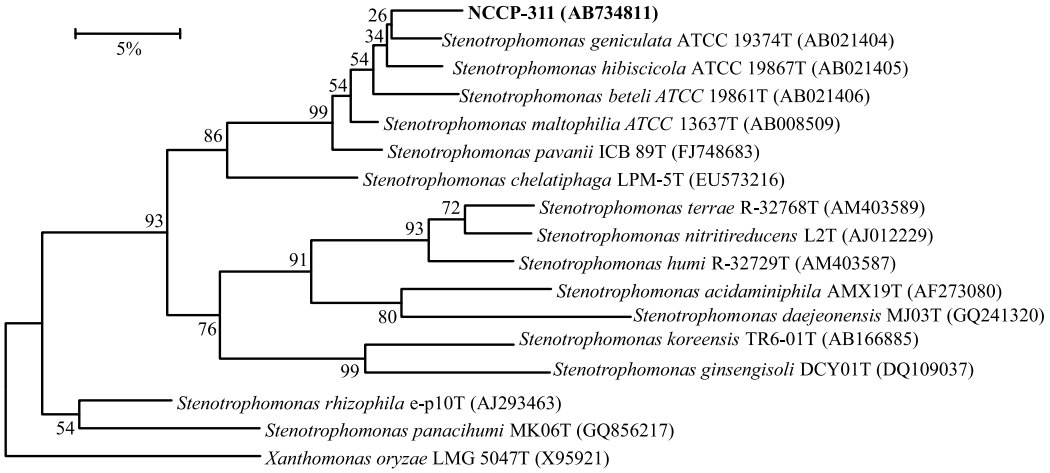

Figure 2. Phylogenetic tree showing the relationships of strain NCCP-311 with the most closely related type species inferred from sequences of 16S rRNA gene. *Xanthomonas oryzae* (X95921) was used as an out group. The tree was generated using the Neighbour-Joining method. Bootstrap values expressed as a percentage of 1000 replications, are given at the branching point. The Bar shows 5% sequence divergence. The accession number of each strain is shown in parenthesis.

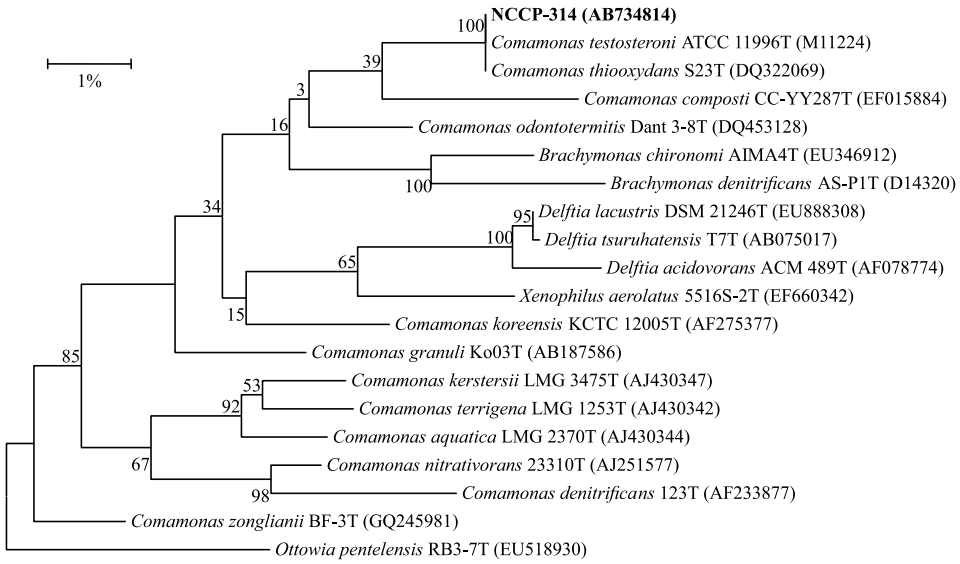

Figure 3. Phylogenetic tree showing relationships strain NCCP-314 with the most closely related species inferred from sequences of 16S rRNA gene. *Ottowia pentelensis* (EU518930) was used as an out group. The tree was generated using the Neighbor-joining method. Bootstrap values expressed as a percentage of 1000 replications, are given at the branching point. The Bar shows 1% sequence divergence. The accession number of each strain is shown in parenthesis.

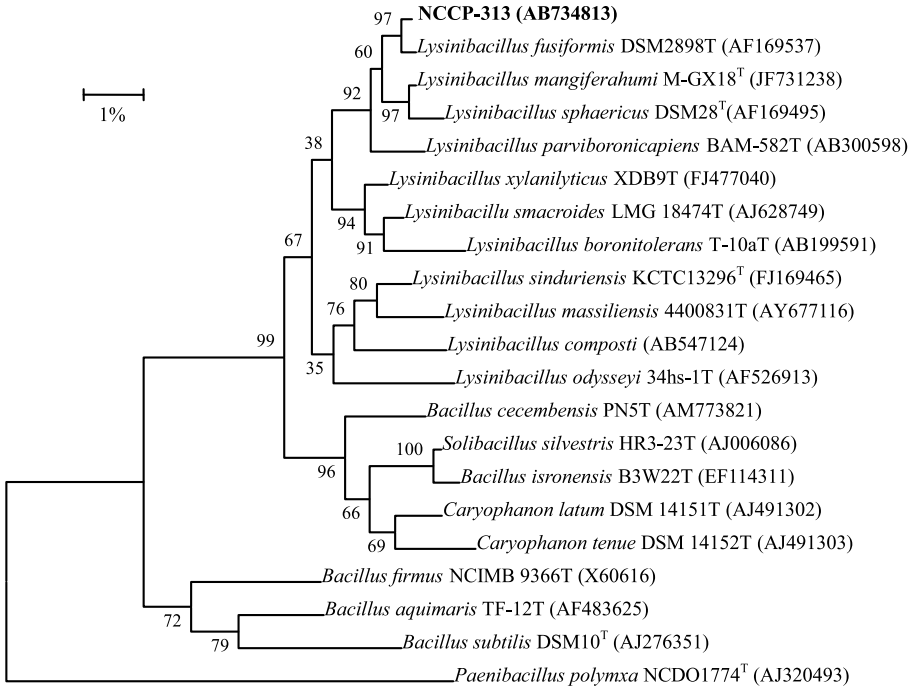

Figure 4. Phylogenetic tree showing the relationships of strain NCCP-313 with the most closely related type species inferred from sequences of 16S rRNA gene. *Paenibacilluspolymxa* (AJ320493) was used as an out group. The tree was generated using the Neighbour-Joining method. Bootstrap values expressed as a percentage of 1000 replications, are given at the branching point. The Bar shows 1% sequence divergence. The accession number of each strain is shown in parenthesis.

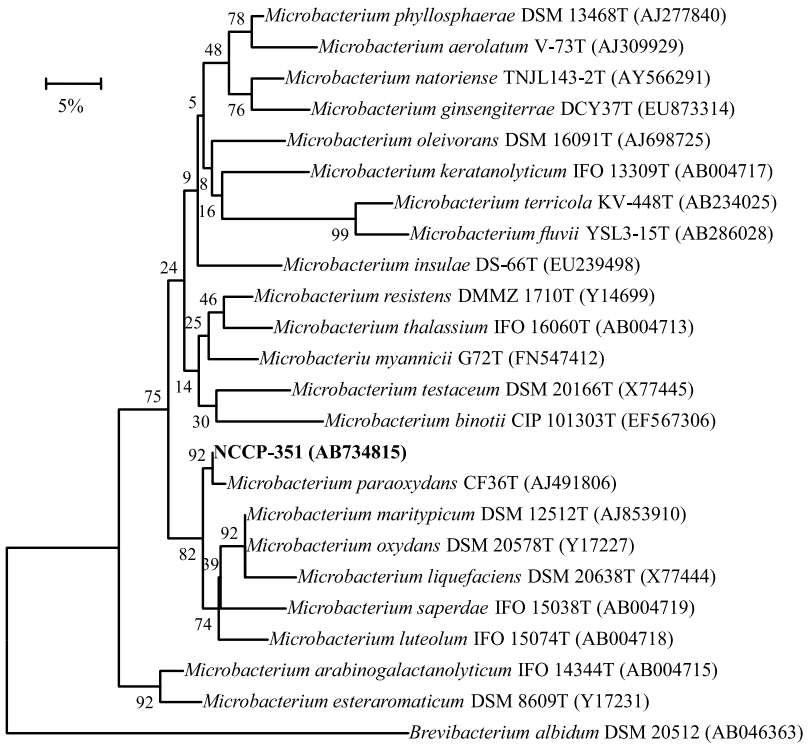

Figure 5. Phylogenetic tree showing the inter-relationships of strain NCCP-351 with the most closely related type species inferred from sequences of 16S rRNA gene. *Brevibacterium albidum* (AB046363) was used as an out group. The tree was generated using the Neighbour-Joining method. Bootstrap values expressed as a percentage of 1000 replications, are given at the branching point. The Bar shows 5% sequence divergence. The accession number of each strain is shown in parenthesis.

and continued up to 47 h. Maximum OD of 0.86 shows the highest growth after 47 h. At 500 mg L^{-1} log phase started from the time of inoculation and continued up to 28 h of inoculation. Maximum growth was observed at 28 h with OD of 0.94 at 600 nm. At 750 mg L^{-1} log phase started from the time of inoculation and continued up to 28 h. Maximum growth was observed at 47 h of inoculation with OD of 1.15. At 1000 mg L^{-1}, log phase was rather slower and started from the time of inoculation and continued up to 47 h. Maximum OD of 0.67 showed highest growth at 47 h (Fig. 6c).

Lysinibacillus sp. NCCP-313 was incubated for a total period of 66 h. At 250 mg L^{-1} phenol concentration, lag phase was observed. Maximum growth (OD 0.49) was observed in log phase that started after 18 h of inoculation and continued up to 48 h and completed in 30 h. At 500 and 750 mg L^{-1} lag phase of 18 h was observed while the log phase continued upto 43 h of inoculation. Maximum OD of 0.57 and 0.65 showing the highest growth was observed after 43 h and 48 h. At 1000 mg L^{-1} phenol concentration no growth was observed (Fig. 6d).

Comamonas sp. NCCP-314 was incubated at 28°C for a total period of 66 h. At 250 mg L^{-1}, lag phase started from the time of inoculation and continued up to 1 day of inoculation. Log phase was started after 1 day of inoculation and continued up to 2 day of inoculation. OD of 0.46 showed the highest growth after 2 days. At 500 and 750 mg L^{-1} no lag phase present, while log phase started from the time of inoculation and continued up to 1 day. Maximum growth was observed at day 1 with OD of 0.591and 0.72 at 600 nm, whereas, no growth was observed at 1000 mg L^{-1} concentration (Fig. 6e).

Microbacterium sp. NCCP-351 was incubated at 28°C for a total period of 120 h. At 250 mg L^{-1} no lag phase was observed, while log phase started from the time of inoculation and continued up to 29 h with maximum OD value of 0.7. At 500 mg L^{-1} phenol concentration there was no lag phase and the log phase prolonged up to 53 h with OD value of 0.55. At 750 mg L^{-1} phenol concentration lag phase started from the time of inoculation and continued up to one day, while log phase took 101 h. The log phase was completed in 77 h with maximum OD of 0.8. At 1000 mg L^{-1} phenol concentration lag phase started from the time of inoculation and continued up to 53 h. Afterwards the log phase completed in 48 with maximum OD of 0.66 (Fig. 6f).

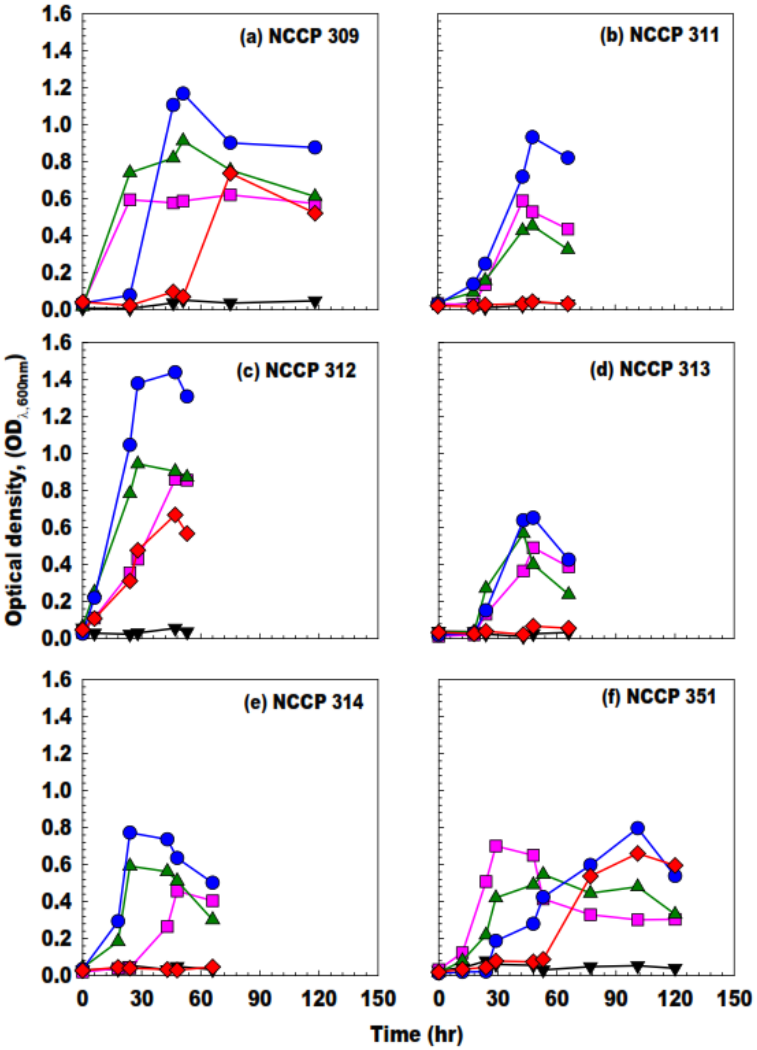

Figure 6. Tolerance of promising strains to different concentration of phenol grown at 28 °C in relation to time scale.
0 mg L^{-1}, 250 mg L^{-1}, 500 mg L^{-1}, 750 mg L^{-1}, 1000 mg L^{-1}.

***Phenol biodegradation*:** Isolated bacteria were studied for their ability to degrade phenol. The strains were grown in MSM supplemented with 750 mg L^{-1} phenol concentration. Optical density was determined from the time of inoculation until stationary phase at different time intervals. Two set of control were used, one with MSM with 750 mg L^{-1} phenol without inoculum and the other had the inoculums without phenol. All the strains showed phenol degradation when supplemented as primary source of carbon and energy. Neither any phenol degradation was observed in control without inoculum nor any growth observed in control without phenol. No lag phase was observed for phenol degradation.

Rhodococcus sp. NCCP-309 degraded 14%, 48% and 96% of phenol (750 mg L^{-1}) in 8, 26 and 32 h, respectively. 100% degradation occurred within 40 h with average degradation rate of 29.1 mg $L^{-1}h^{-1}$ (Fig. 7a). *Stenotrophomonas* sp. NCCP-311 took longer time and degraded 8%, 21% 29%, 77%, and 89% of phenol in 23, 28, 32, 47 and 60 h, respectively. This strain degraded 100% of 750 mg L^{-1} phenol within 71 h with an average degradation rate of 16 mg $L^{-1}h^{-1}$ (Fig. 7b). *Rhodococcus* sp. NCCP-312 degraded 12, 35 56, 64 and 99.6% of phenol in 8, 26, 32, 49 and 53 h, respectively. The strain degraded 100% phenol within 56 h with an average degradation rate of 12.5 mg $L^{-1}h^{-1}$ (Fig. 7c). *Lysinibacillus* sp. NCCP-313 degraded 9, 24, 25, 69, 75, 88 and 99.6% of phenol (750 mg L^{-1}) in 6, 23, 28, 32, 47, 71 and 78 h, respectively. The strain degraded 100% of 750 mg L^{-1} phenol within 96 h with an average degradation rate of 22.7 mg $L^{-1}h^{-1}$ (Fig. 7d).

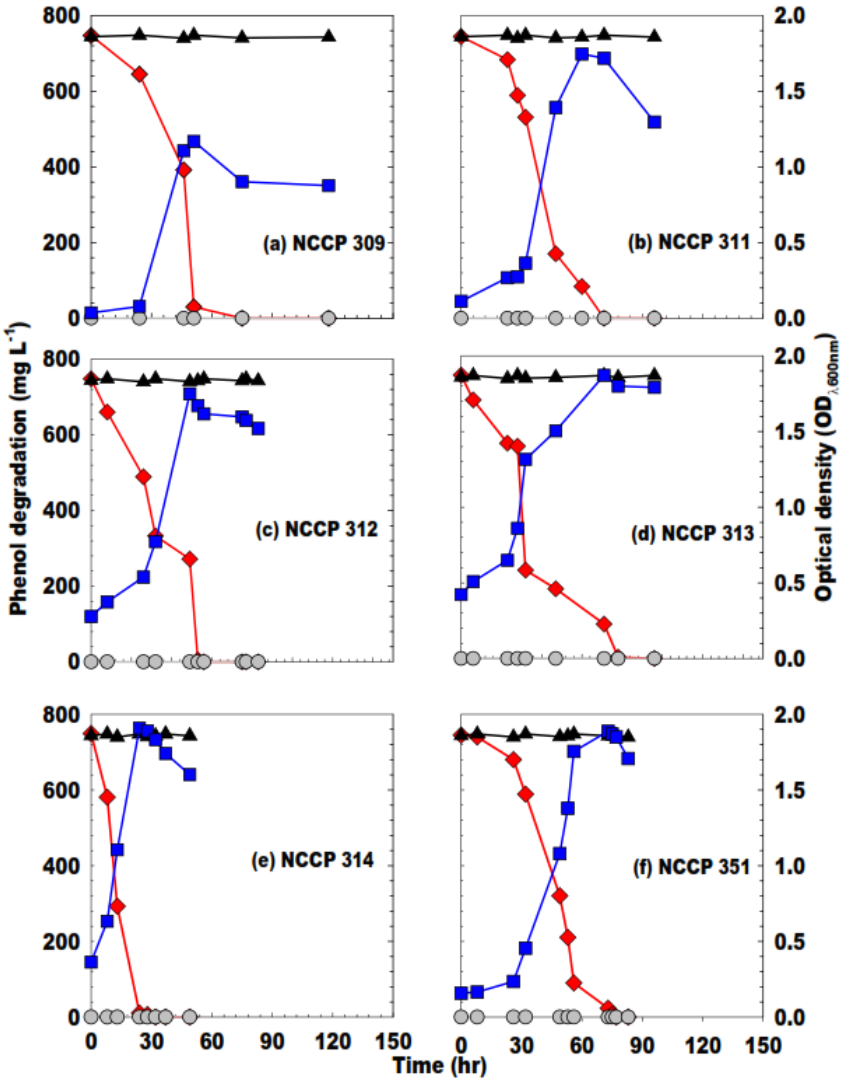

Figure 7. Degradation of phenol (a – f) and growth response as measured by optical density (a – f). phenol concentration in inoculum, phenol concentration in control without inoculum, observed $OD_{\lambda,600}$, control $OD_{\lambda,600}$.

Comamonas sp. NCCP-314 degraded 22, 61, 98.6 and 99% of phenol in 8, 13, 24 and 32 h, respectively. For this strain, 100% phenol degradation took place within 32 h with an average degradation rate of 34.8 mg $L^{-1}h^{-1}$ (Fig. 5e). *Microbacterium* sp. NCCP-351 degraded 9, 21, 57, 72, 88, 97 and 99% of phenol (750 mg L^{-1}) in 26, 32, 29, 53, 56, 73 and 75 h, respectively. The strain degraded 100% of 750 mg L^{-1} phenol within 77 h with an average degradation rate of 24.7 mg $L^{-1}h^{-1}$ (Fig. 7f).

DISCUSSION

Microorganisms have the ability to utilize toxic organic compounds such as phenol at lower concentrations, because they need a carbon source for their metabolic processes (Cokgor *et al.*, 2008). Phenol degrading bacteria belongs to different genera and mostly include *Pseudomonas*, *Agrobacterium*, *Acinetobacter*, *Klebsiella*, *Bacillus*, *Rhodococcus* and *Rhizobium* (Koutny *et al.*, 2003). The present work describes the isolation and characterization of indigenous phenol degradation bacterial strains from highly contaminated industrial wastes. The industrial waste was collected from two different areas of Pakistan and isolated on MSM media previously enriched with phenol. Many reports have shown that enrichment of bacterial strains is necessary for degradation of phenol (Balk *et al.*, 2010). In

this study eight morphologically different strains were identified on the basis of 16S rRNA gene sequence analysis. Among the isolated strains, *Rhodococcus* sp. NCCP-309, *Rhodococcus* sp. NCCP-312, *Comamonas* sp. NCCP-314, *Stenotrophomonas* sp. NCCP-311, *Lysinibacillus* sp. NCCP-313 and *Microbacterium* sp. NCCP-351 were further evaluated for phenol degradation tests because the genus *Rhodococcus* and *Comamonas* are well known for bioremediation as these genera exhibit a wide range of enzyme activities. The species in these genera have ability to grow on a variety of complex carbon sources, such as short- and long-chain as well as halogenated hydrocarbons, aromatic compounds, polycyclic aromatic compounds, and steroids (Koutny *et al.,* 2003; Shen *et al.*, 2009). Yoon *et al.* (2000) identified and reported a novel *Rhodococcus pyridinivorans* strain capable of pyridine degradation.

Based on our results, *Rhodococcus* sp. NCCP-309 shared 98.9% sequence similarity of 16S rRNA gene with *Rhodococcus phenolicus* (AM933579) (Table 1; Fig. 1), which was isolated by Johnson Space Center and reported to have phenol (0.75% w/v) degrading ability (Rehfuss and Urban, 2005). The 16S rRNA gene sequence similarity greater than 98.5% among representatives of *Rhodococcus* species can be considered as novel species (Yassin, 2005) because of sharing whole genomic relatedness values well below the 70% cut-off point recommended for the delineation of bacterial species (Wayne *et al*., 1987). The sequence similarity of NCCP-309 of 98.9% with this species may give a further opportunity to investigate NCCP-309 taxonomically for delineation of possible novel species; however, the taxonomic studies are beyond the scope of this manuscript.

On the basis of these results, NCCP-309 may be different than the previously reported *Rhodococcus phenolicus* in terms of higher degradation ability and significant growth on enriched medium. Our isolated strain and *Rhodococcus phenolicus* showed positive result for *β*-galactosidase but negative for H_2S production, urease, fermentation of glucose, mannitol and sorbitol. In comparison *Rhodococcus* NCCP-312 shared 99.93% 16S rRNA sequence similarity with *Rhodococcus zopfii* (AF191343) which was also reported for phenol degradation. *Rhodococcus* NCCP-312 was negative for all the biochemical substrates (Table 2) and the reference strains *Rhodococcus zopfii* (Stoecker *et al*., 1994) is positive for *β*-galactosidase and urease.

Comamonas NCCP-314 and *Stenotrophomonas* NCCP-311 shared 100% and 99.64% similarity, respectively with *Comamonas thiooxidans* (DQ322069) and *Stenotrophomonas geniculata* (AB021404). Members of genus *Comamonas* are reported for the degradation of different phenol derivatives (Chen *et al.,* 2003). Similarly, strains belonging to *Stenotrophomonas* are also reported for degradation of various xenobiotics. Papizadeh *et al*. (2011) reported the isolation of *Stenotrophomona*s sp. NISOC-04 which degraded dibenzothiophene, whereas Liu *et al*. (2007) reported that *Stenotrophomonas* LZ-1 shows growth on p-nitrophenol and 4-chlorophenol. There has been no previous report on the phenol degradation ability of *Stenotrophomonas* and our study is therefore, the first report that describes phenol degrading potential of *Stenotrophomonas* at different concentration of phenols.

***Phenol degrading ability of Rhodococcus, Comamonas Stenotrophomonas, Lysinibacillus and Microbacterium*:** From various environments, members of the genus *Rhodococcus* are isolated with diverse physiological and morphological characteristics and many members of the *Rhodococcus* are reported for phenol degradation (Naiem and Ghosh, 2011). In this study *Rhodococcus* NCCP-309 and *Rhodococcus* NCCP-312 degraded 750 mg L^{-1} of phenol with the average degradation rate of 29.1 mg $L^{-1}h^{-1}$ and 12.5 mg $L^{-1}h^{-1}$, respectively which showed that the *Rhodococcus* NCCP-309 degraded phenol faster than *Rhodococcus* NCCP-312. Similarly, NCCP-309 strain showed growth at all given concentrations of phenol, while 1000 mg L^{-1} of phenol was toxic for NCCP-312. Increase in degradation rate could be attributed to an increase in the substrate availability for cell growth (Yoon *et al.,* 2000) and decrease in the concentration of phenol is coincided with the increase in growth as indicated by increase in OD. Our results are in accordance with Shumkova *et al.* (2009) with a little deviation, they reported that the strain *Rhodococcus opacus* Strain 1G efficiently degraded 750 mg L^{-1} of phenol in about 20 h. Similarly, Suhaila *et al*. (2010) observed that *Rhodococcus* UKM-P degraded 500 mg L^{-1} of phenol in 21 h. The variation in phenol degrading time might be acceptable because within genus *Rhodococcus*, different strains can degrade different organic compounds even for single substrate utilization (Larkin *et al*., 2005).

Comamonas NCCP-314 degraded 750 mg L^{-1} of phenol with an average degradation rate of 34.8 mg $L^{-1}h^{-1}$. The degradation completed in 32 h which was fastest among all the isolated strains. The obtained results are in line with those of Chen *et al.* (2003); their strain *Comamonas testosterone* ZD 4-1 tolerated 500 mg L^{-1} of phenol and degraded it in 48 h. Similar results were reported by Arai *et al*. (1998) with *Comamonas testosterone*. Although 16S rRNA sequence showed similarity with Chen *et al.* (2003) but our isolated strain showed more degradation in comparison with Chen *et al*. (2003) and Arai *et al.* (1998).

In comparison, the growth of *Stenotrophomonas* NCCP-311 at 750 mg L^{-1} of phenol was less than that of *Rhodococcus* NCCP-309 and *Rhodococcus* NCCP-312**.** The time taken by *Stenotrophomonas* NCCP-311 for 750 mg L^{-1} of phenol degradation was more than that of all the isolated strains. The present results are in line with study of Liu *et al.* (2007); their strain LZ-1 removed p-nitrophenol and 4-chlorophenol within 14 and 16 days respectively. Similarly,

Stenotrophomonas sp. NISOC-04 had ability to degrade dibenzothiophene (Papizadeh *et al*., 2011).

Lysinibacillus NCCP-313 degraded 750 mg L^{-1} phenol in 96 h which was more than other characterized strains. This strain shares 99.87% 16S rRNA sequence similarity with *Lysinibacillus sphaericus* (AF169495) which is not previously reported for phenol degradation but is capable of p-nitrophenol oxidation (Kadiyala *et al.*, 1998). The strain *Lysinibacillus* NCCP-313 showed similarity with *Lysinibacillus sphaericus* (AF169495) (Claus and Berkeley, 1986) at molecular level as well as physiologically as both strains showed positive results for gelatinase and negative for urease, tryptophane deaminase, sodium pyruvate, fermentation/oxidation of saccharose and NO_2 production.

Microbacterium species were isolated from diverse sources like water, plant, air, wastes, insect and sludge etc (Evtushenko and Takeuchi, 2006). The strain shares 99.15% 16S rRNA sequence similarity with *Microbacterium paraoxydans* (AJ491806), which is not reported for phenol degradation. *Microbacterium* NCCP-351 is different from *Microbacterium paraoxydons* (Laffineur *et al.*, 2003) as *Microbacterium* NCCP-351 showed negative results for fermentation/oxidation glucose, rhamnose and arabinose and *Microbacterium paraoxydons* is positive for all the above substrates**.**

***Conclusion*:** The identified bacterial strains of *Rhodococcus, Comamonas* and *Stenotrophomonas* can be used for active phenol biodegradation in a bioreactor. *Rhodococcus* sp. NCCP-309 and *Rhodococcus* sp. NCCP-312 tolerated resistance up to 1000 mg L^{-1} of phenol, while *Comamonas* and *Stenotrophomonas* strains toxicity of phenol up to 750 mg L^{-1} phenol. The phenol degrading rate of *Rhodococcus* sp. NCCP-309 was faster than that of *Rhodococcus* sp. NCCP-312 in comparison to other strains. The isolated strains can be used for bioremediation of phenol in highly contaminated industrial areas.

***Acknowledgments*:** This work was supported by financial assistance from PSDP funded Project *Research for Agricultural Development Project* under a sub-project (Grant No. CS-55/RADP/PARC) entitled “Establishment of Microbial Bio-Resource Laboratories: National Culture Collection of Pakistan (NCCP)” from Pakistan Agricultural Research Council, Islamabad, Pakistan

REFERENCES

Agency for Toxic Substances and Disease Registry (ATSDR). 2011**.** Toxico-logical profile for phenol. In. Atlanta: Public Health Services, U.S. Department of Health and Human Services.

Ahmad, N., I. Ahmed, A. Shahzad, N. Khalid, F. Mehboob, K. Ahad and G.M. Ali. 2014. Molecular identification and characterization of *Pseudomonas* sp. NCCP-407 for phenol degradation isolated from industrial waste. J. Korean Soc. Appl. Biol. Chem. 57: 341-346.

Ahmad, S., M. Syed, N. Arif, M. Shukor and N. Shamaan. 2011**.** Isolation, identification and characterization of elevated phenol degrading *Acinetobacter* sp. strain. Aust. J. Basic Appl. Sci. 5:1035-1045.

Ahmed, I., A. Yokota and T. Fujiwara. 2007**.** A novel highly boron tolerant bacterium, *Bacillus boroniphilus* sp. nov., isolated from soil, that requires boron for its growth. Extremophiles 11:217–224.

Alber, M., H.B. Bohm, J. Brodesser, J. Feltes, K. Levsen and H.F. Schöler. 1989**.** Determination of nitrophenols in rain and snow. Z. Anal. Chem. 334: 540-545.

Arai, H., S. Akahira, T. Ohishi, M. Maeda and T. Kudo. 1998**.** Adaptation of *Cornamonas testosteroni* TAM1 to utilize phenol: organization and regulation of the genes involved in phenol degradation. Microbiol. 144: 2895-2903.

Bai, J., J.-P. Wen, H.-M. Li and Y. Jiang. 2007**.** Kinetic modeling of growth and biodegradation of phenol and *m*-cresol using *Alcaligenes faecalis*. Process Biochem. 42: 510-517.

Balk, M., F. Mehboob, A. Gelder, W.I. Rijpstra, J.S. Damsté and A.M. Stams. 2010**.** (Per)chlorate reduction by an acetogenic bacterium, *Sporomusa* sp., isolated from an underground gas storage. Appl. Microbiol. Biotechnol. 88: 595-603.

Banerjee, A. and A.K. Ghoshal. 2010**.** Phenol degradation by *Bacillus cereus*: Pathway and kinetic modeling. Bioresource Technol. 101: 5501-5507.

Basha, K., A. Rajendran and V. Thangavelu. 2010**.** Recent advances in the biodegradation of phenol. Asian J. Experim. Biol. Sci. 1:219-234.

Brown, J.A. 2008**.** An internet database for the classification and dissemination of information about hazardous chemicals and occupational diseases. Am. J. Ind. Med. 51:428-435.

Cao, J., C. Dong, H. Cao and Z. Shao. 2011**.** Isolation of phenol degrading bacteria from coking wastewater and their degradation gene. Huan jing ke xue= Huanjing kexue / [bian ji, Zhongguo ke xue yuan huan jing ke xue wei yuan hui "Huan jing ke xue" bian ji wei yuan hui.] 32:560-566.

Carmona, M., A.D. Lucas, J.L. Valverde, B. Velasco and J.F. Rodríguez. 2006**.** Combined adsorption and ion exchange equilibrium of phenol on Amberlite IRA-420. Chem. Eng. J. 117:155-160.

Chen, K.-C., J.-Y. Wu, D.-J. Liou and S.-C.J. Hwang. 2003**.** Decolorization of the textile dyes by newly isolated bacterial strains. J. Biotechnol. 101:57-68.

Cokgor, E.U., O. Karahan and D. Orhon. 2008**.** The effect of mixing pharmaceutical and tannery wastewaters on the

biodegradation characteristics of the effluents. J. Hazardous Mat. 156:292-299.

Das, S. and S. Santra. 2012**.** Phenol removal from coal refining wastewater by immobilized cyanobacteria. Electron. J. Environ. Agri. Food Chem. 11:673-679.

Ehrt, S., F. Schirmer and W. Hillen. 1995**.** Genetic organization, nucleotide sequence and regulation of expression of genes encoding phenol hydroxylase and catechol 1,2-dioxygenase in *Acinetobacter calcoaceticus* NCIB8250. Mol. Microbiol. 18:13-20.

El-Sayed, W., M. Ibrahim, M. Abu-Shady, F. El-Beih, N. Ohmura, H. Saiki and A. Ando. 2003**.** Isolation and characterization of phenol-catabolizing bacteria from a coking plant. Biosci. Biotechnol. Biochem. 67:2026-2029.

Felsenstein, J. 2005**.** PHYLIP (Phylogeny Inference Package). Version 3.6. distributed by the author. Department of Genome Sciences, University of Washington,: Seattle, Washington.

Giti, E., H. Mehdi and G. Nasser. 2005**.** Development of a microtitre plate method for determination of phenol utilization, biofilm formation and respiratory activity by environmental bacterial isolates. Int. Biodeter. Biodegr. 56:231-235.

Hall, T. A. 1999**.** BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT: Nucleic Acids Symposium Series.

Koutny, M., J. Ruzicka and J. Chlachula. 2003**.** Screening for phenol-degrading bacteria in the pristine soils of south Siberia. Appl. Soil Ecol. 23:79-83.

Larkin, M.J., L.A. Kulakov and C.C.R. Allen. 2005**.** Biodegradation and *Rhodococcus* – masters of catabolic versatility. Curr. Opin. Biotechnol. 16:282-290.

Lazarova, Z. and S. Boyadzhieva. 2004**.** Treatment of phenol-containing aqueous solutions by membrane-based solvent extraction in coupled ultrafiltration modules. Chem. Eng. J. 100:129-138.

Liu, Z., C. Yang and C. Qiao. 2007**.** Biodegradation of p-nitrophenol and 4-chlorophenol by *Stenotrophomonas* sp. FEMS Microbiol. Lett. 277:150-156.

Loh, K.-C. and S.-S. Chua. 2002**.** Ortho pathway of benzoate degradation in *Pseudomonas putida*: induction of meta pathway at high substrate concentrations. Enz. Microbial Technol. 30:620-626.

Lowry, M., A. Nagamani, K. Sreenivasulu and R. Soligalla. 2009**.** Isolation and characterization of phenol-degrading soil bacterium *Xanthobacter flavus*. Bioremed. J. 13:1-6.

Mahiudddin, M., A.N.M. Fakhruddin and Abdullah-Al-Mahin. 2012**.** Degradation of phenol via meta cleavage pathway by *Pseudomonas fluorescens* PU1. ISRN Microbiol. 2012:1-6.

Mohite, B., R. Jalgaonwala, S. Pawar and A. Morankar. 2010**.** Isolation and characterization of phenol degrading bacteria from oil contaminated soil. Inn Roman Food Biotechnol. 7:61-65.

Movahedyan, H., H. Khorsandi, R. Salehi and M. Nikaeen. 2009**.** Detection of phenol degrading bacteria and *Pseudomonas putida* in activated sludge by polymerase chain reaction. Iran J. Environ. Health Sci. Eng. 6:115-120.

Naiem, H. and J. Ghosh. 2011**.** Purification and characterization of catechol 1, 2-dioxygenase from *Rhodococcus* sp. NCIM 2891. . Res. J. Environ. Earth Sci. 3:608-613.

Nair, C., K. Jayachandran and S. Shashidhar. 2008**.** Biodegradation of phenol. African J Biotechnol. 7:4951-4958.

Naresh, B., P. Honey and S. Vaishali. 2012**.** Biodegradation of phenol by a bacterial strain isolated from a phenol contaminated site in India. Ind. Res. J. Environ Sci. 1:46-49.

Nuhoglu, A. and B. Yalcin. 2005**.** Modelling of phenol removal in a batch reactor. Process Biochem. 40:1233-1239.

Papizadeh, M., M. Ardakani, H. Motamedi, I. Rasouli and M. Zarei. 2011**.** C–S targeted biodegradation of dibenzothiophene by *Stenotrophomonas* sp. NISOC-04. Appl. Biochem. Biotechnol. 165: 938-948.

Park, J.-S., M.T. Brown and T. Han. 2012**.** Phenol toxicity to the aquatic macrophyte Lemna paucicostata. Aquatic Toxicol. 106–107: 182-188.

Pradeep, N.V., Anupama and U. Hampannavar. 2011**.** Biodegradation of phenol using rotating biological contactor. Int. J. Environ. Sci. 2:105-113.

Rehfuss, M. and J. Urban. 2005**.** *Rhodococcus phenolicus* sp. nov., a novel bioprocessor isolated actinomycete with the ability to degrade chlorobenzene, dichlorobenzene and phenol as sole carbon sources. Syst. Appl. Microbiol. 28:695-701.

Sandhu, A., L. Halverson and G. Beattie. 2009**.** Identification and genetic characterization of phenol-degrading bacteria from leaf microbial communities. Microb Ecol. 57:276-285.

Shen, F.-T., J.-L. Lin, C.-C. Huang, Y.-N. Ho, A.B. Arun, L.-S. Young and C.-C. Young. 2009**.** Molecular detection and phylogenetic analysis of the catechol 1,2-dioxygenase gene from *Gordonia* spp. Syst. Appl. Microbiol. 32:291-300.

Shumkova, E., I. Solyanikova, E. Plotnikova and L. Golovleva. 2009**.** Phenol degradation by *Rhodococcus opacus* Strain 1G. Appl. Biochem. Microbiol. 45:51-57.

Steiner, F., F. McLeod, J. Rohrer, S. Fabel, L. Lang, X. Qun and C. Jing. 2008**.** Determination of phenols in drinking and bottled mineral waters using online solid-phase extraction followed by HPLC with UV Detection. Germany: Dionex Corporation.

Stoecker, M.A., R.P. Herwig and J.T. Staley. 1994**.** *Rhodococcus zopfii* sp. nov., a toxicant-degrading bacterium. Int. J. Syst. Bacteriol. 44:106-110.

Suhaila, N., A. Ariff, M. Rosfarizan, A.I. Latif, S.A. Ahmad, M.N. Norazah and M.Y.A. Shukor. 2010**.** Optimization of parameters for phenol degradation by *Rhodococcus* ukm-p in shake flask culture. World Congress on Engineering United Kingdom.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar. 2011**.** MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28:2731-2739.

Thompson, J.D., D.G. Higgins and T.J. Gibson. 1994**.** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acid Res. 22:4673-4680.

Tsao, C., H. Song and R. Bartha. 1998**.** Metabolism of benzene, toluene, and xylene hydrocarbons in soil. Appl. Environ. Microbiol. 64:4924-4929.

Tuah , P.M., N.A.A. Rashid and M.M. Salleh. 2009**.** Degradation pathway of phenol through ortho-cleavage by *Candida tropicalis* Retl-Cr1. Borneo Sci. 24:1-8.

US-EPA. 1979**.** Phenol ambient water quality criteria. Washington, DC: National Service Center for Environmental Publications (NSCEP), Criteria and Standards Division, Office of Water Planning and Standards, US Environmental Protection Agency, Washington, DC.

Wayne, L.G., D.J. Brenner, R.R. Colwell and other authors. 1987**.** Report of the adhoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. 37:463-464.

Wu, J., K. Rudy and J. Spark. 2000**.** Oxidation of aqueous phenol by ozone and peroxidase. Adv. Environ. Res. 4: 339-346.

Yan, J., W. Jianping, B. Jing, W. Daoquan and H. Zongding. 2006**.** Phenol biodegradation by the yeast *Candida tropicalis* in the presence of m-cresol. Biochem Eng J. 29:227-234.

Yassin, A.F. 2005**.** *Rhodococcus triatomae* sp. nov., isolated from a blood-sucking bug. Int. J. Syst. Evol. Microbiol. 55:1575-1579.

Yoon, J.H., S.S. Kang, Y.G. Cho, S.T. Lee, Y.H. Kho, C.J. Kim and Y.H. Park. 2000**.** *Rhodococcus pyridinivorans* sp. nov., a pyridine-degrading bacterium. Int. J. Syst. Evol. Microbiol. 50:2173-2180.

Zhai, Z., H. Wang, S. Yan and J. Yao. 2012**.** Biodegradation of phenol at high concentration by a novel bacterium: *Gulosibacter* sp. YZ4. J. Chem. Technol. Biotechnol. 87:105-111.