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Characterization of Phenol Degrading Bacteria Isolated from Industrial Effluents

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ABSTRACT

In this study, phenol degrading activity by bacterial isolates collected from industrial effluents was investigated. Isolates were identified as *Stenotrophomonas maltophilia* and *Bacillus subtilis* by 16S rRNA analysis. Both isolates showed maximum survival in the presence of phenol up to 300 µg/mL. They were aerobic bacteria that exhibited maximum growth in the presence of phenol at 37°C, pH 7. Among intracellular and extracellular enzyme activity, intracellular was found comparatively predominant. The intracellular enzyme activity was observed under wide ranges of pH (5-9), temperature (30°C-90°C) and metal ions (Na^+, Mg^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+}). Maximum enzyme activity was observed at pH 5 and temperature at 90°C. HPLC studies confirmed that both bacterial species possess the efficient ability of phenol degradation. Conclusively, above mentioned bacteria can be exploited for the removal of phenol from polluted water or land, which will be cost effective and safe as well.

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Authors' Contribution
AR designed the study and analyzed the data. MR performed the experiments. AR and MR wrote the article.

Key words
Biodegradation, phenol, wastewater, *Bacillus subtilis*, *Stenotrophomonas maltophilia*

INTRODUCTION

Phenol is included in the group of organic pollutants that are considered hazardous as it can harm organisms including human beings even at very low concentrations (Lin and Wu, 2011). Phenol is a widely employed chemical in different industries such as agriculture chemical, textile, plastic, paper and pulp, wood preservation, oil refineries, pharmaceuticals, wine distilleries and coal coking (Kashif and Ouyang, 2009; Levén and Schnürer, 2010) and thus the part of their waste. Phenolic compounds are not only released in the industrial effluents but also exist naturally in some compounds such as tea and wood. It is also added to the environment in the form of byproduct formed during the combustion of fossil fuels and organic waste of animals (Busca *et al*., 2008). Usually, the source of contaminating aquatic and terrestrial biota is the industrial waste released directly into water bodies or land. When ingested through drinking water or smoked food phenol badly affects lungs, kidneys, liver and vascular system (Harris *et al*., 2005). To protect our environment and ultimately human lives from adverse effects of phenolic compounds, wastewater should be cleaned before release (Kashif and Ouyang, 2009). But the problem is there that phenol is resistant to degradation and it cannot be converted to nontoxic forms, hence making the reclamation of the affected areas more and more difficult (Shourian *et al*., 2009).

There are many methods applied for the removal of phenolic compounds from the wastewaters released from the industries. These may include air stripping, bioadsorption, chemical oxidation (chlorine dioxide, hydrogen peroxide, ozone), deep well injection, irradiation, solvent extraction and incineration (Kulkarni and Kaware, 2013). Methods like solvent extraction, adsorption and oxidation are very much expensive, while deep well injection may contaminate ground water (Aksu, 2005).

The most efficient method being conventionally used for the removal of phenol and its derivatives from wastewaters is the use of microorganisms (Rosenkranz *et al*., 2013). Microorganisms in use are bacterial cultures (*Pseudomonas*, *Bacillus*) or different fungal and yeast species (*Penicillium*, *Aspergillus*, *Candida*, *Trichosporon*) (Vallini *et al*., 2001; Mohite *et al*., 2011; Bonfá *et al*., 2013). They degrade phenol either directly by using it as a sole carbon source or indirectly in the presence of any other growth substrate by a process called co-metabolism (Pradhan and Ingle, 2007). Microorganisms produce a variety of enzymes which have the ability to act on phenol and its derivatives. The enzymes produced for degradation of phenol by microorganisms are intracellular as well as extracellular oxidases (Vaillancourt *et al*., 2006).

Many of the industries producing phenol and its derivatives are located in the suburbs of Lahore, Pakistan. This has promoted the initiative to conduct this study. Isolates obtained from industrial waste were identified and characterized under standard laboratory conditions. Moreover, degradation of phenol was estimated by analyzing the type of enzyme produced by these isolates.

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Protein estimation and purification study was done by High Performance Liquid Chromatography (HPLC).

MATERIALS AND METHODS

Enrichment and isolation of phenol degrading bacteria

Bacteria capable of degrading phenol were isolated from seven samples of industrial wastewater collected from paper and leather industries, Sheikhupura. Water samples were then used to prepare serial dilutions from 10^{-2} to 10^{-6}. Then 100 µL from each dilution was inoculated in respective flask containing 50 mL Minimal Salt (MS) medium (prepared by K_2HPO_4 1.36 g/L, Na_2HPO_4 1.42 g/L, $MgSO_4.7H_2O$ 0.12 g/L, $CaCl_2$ 0.006 g/L, glucose 10 g/L, yeast extract 10 g/L, tryptone 10 g/L and 1mL/L trace mineral solution) and 100 µg/mL of phenol as a carbon source. After 5 days of incubation at 37°C, 500 µL from each flask was transferred to respective flasks of MS medium with 200 µg/mL of phenol as a sole carbon source. Similar procedure was repeated for third time with increasing the concentration of phenol up to 300 µg/mL and dilutions were plated on MS agar plates containing 300 µg phenol/mL. Resulting colonies were streaked again on MS agar plates to get pure cultures.

Molecular identification

Total DNA of two bacterial isolates 6-RM and S-RM was extracted as described by Andreou (2013) and amplification of 16S rRNA gene was done by universal primers. The conditions followed for amplification were as follows: Initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 57°C for 1 min, extension at 72°C for 1.5 min and final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis in 1% agarose gel in 1X TAE buffer with ethidium bromide and visualized under UV transilluminator. Purified PCR products were sent to CEMB, Thokar Niaz Baig, Lahore for sequencing.

Determination of optimum growth conditions

To evaluate optimum growth temperature and pH for the strains selected after screening, method by (Lee and Wang, 2004) was adopted. N-broth was prepared and autoclaved. After cooling it was poured into two sets of twelve tubes. In each set three tubes were inoculated with freshly prepared culture of isolate 6-RM and S-RM, respectively while four tubes in each set were not inoculated and served as control. All tubes were incubated at 20°C, 30°C, 37°C, 45°C and absorbance was taken after 24 h at 600 nm. Similarly, for determination of optimum pH for 6-RM and S-RM two sets of 18 tubes containing 5 mL LB each were prepared. The pH was maintained at 5, 6, 7, 8, 9 and 10 by using NaOH and HCl. In each set 6 tubes served as control and others were inoculated with 30 µL of freshly prepared cultures of 6-RM and S-RM. The tubes were incubated at 37°C for 24 h and absorbance was measured at 600 nm.

Degradation of phenol by bacterial strains

Each of the isolate was cultivated in 50 mL LB medium and put on rotary shaker at 37°C till the cells were at log phase. Bacterial culture was harvested and pellet was dissolved in phosphate buffer (pH 7) with adjusted OD_{600} of 1.0. Three 250 mL Erlenmeyer flasks containing 100 mL of MS medium supplemented with phenol (100 µg/mL) as carbon source were prepared. Isolates, S-RM and 6-RM, were inoculated 1.5% v/v to the two flasks containing media while other served as control. Flasks were incubated at 37°C and change in growth was measured periodically at $OD_{600\ nm}$.

Enzyme assays

Isolate 6-RM and S-RM were grown in 100 mL of MS medium for 24 h. After 24 h of incubation phenol was added at a concentration of 100 µg/mL and flasks were again incubated for 4-5 days. After 5 days cells were harvested and pellets were separated from supernatants. Both were used separately to determine the extracellular and intracellular nature of the enzyme. Each experiment was performed in triplicate.

Intracellular enzyme assay

Cells were suspended in 100 mM sodium phosphate buffer (PBS) pH 7 and were sonicated at maximum power (twice for 20 sec with 1 min interval). Cell extract (600 µL) was added to the mixture of 2 mL 100 mM PBS, 300 µL 40 mM EDTA and 30 µL of 30 mM catechol. The presence of Catechol-1,2-dioxygenase (C12D) or Catechol-2,3-dioxygenase (C23D) was detected by the formation of products *cis-cis* muconic acid and *2*-hydroxymuconic semialdehyde (2-HMS) at 260 nm and 375 nm, respectively (Santos and Linardi, 2004). Assay mixture was incubated at 30°C for 25 min before spectrophotometry.

Extracellular enzyme assay

For this purpose, 70% (w/v) chilled ammonium sulfate was added to the supernatant and left overnight at 4°C. The solution was then centrifuged at 6000 rpm for 10 min and supernatant was used as crude enzyme. This crude enzyme was used in the same manner as discussed above and absorbance was taken at 260 nm and 375 nm.

C23D activity as a function of temperature, pH and metal ions

Change in enzyme activity was measured by incubating crude enzyme at temperatures 30°C-90°C for 25 min. After the treatment absorbance was recorded for residual enzyme activity.

Effect of pH on the enzyme activity was determined under standard assay conditions using buffers: Sodium acetate 0.1 M (pH 5-6), sodium phosphate 0.1 M (pH 7-8), Tris-HCl 0.1 M (pH 9). Standard was prepared in sodium phosphate buffer 0.1 M (pH 7). All were incubated at 25°C and absorbance was taken at 375 nm (Aydemir, 2004).

Stock solutions of different metals (NaCl, $MgCl_2$, $MnCl_2$, $ZnCl_2$ and $CuSO_4$) were made with the concentration 1mM/mL in the phosphate buffer. Two mL of each metal salt was added in the reaction mixture for each isolate and enzyme activity was measured by taking absorbance at 375 nm. Control reaction mixture contained phosphate buffer instead of metal ion solution.

High Performance Liquid Chromatography (HPLC)

Phenol standard (0.5M) and crude enzyme samples from the two isolates were examined by using HPLC, equipped with C_{18} column. Samples were eluted at a flow rate of 1 mL/min with mobile phase methanol and water mixed in a ratio 4:3. A UV detector was used to analyze the samples at 375 nm (Michizoe *et al*., 2005).

Statistical analysis

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. Each time three readings were taken, their mean, and standard error of the mean were calculated.

RESULTS AND DISCUSSION

Physiological and molecular characterization

Phenol degrading enzymes are widely distributed in different organisms such as bacteria, fungi and algae and all of them are believed to play an important role in the degradation of phenol (Haritash and Kaushik, 2009). Until now a large number of bacterial strains have screened for this activity. The present work describes the isolation of local strains, 7 samples of industrial wastewater, capable of degrading phenol and using it as carbon source. We have applied enrichment method for isolation to obtain specific bacteria among diverse natural population. These enrichments have allowed the adaptation of bacteria to 300 µg/mL of phenol in MS medium. Growth was negligible on applying higher concentration of phenol. This may had occurred due to sensitivity of these bacteria towards higher concentration of phenol or they may require adaptation to it prior starting its degradation (Abd-El-Haleem *et al*., 2002). It was further emphasized when these two isolates were separately grown in MS medium and MS medium supplemented with phenol. Periodic observation of growth in the medium with phenol showed that bacteria gradually adapt themselves to the compound (Fig. 1). Optimum temperature and pH for growth of these two isolates were observed at 37°C and pH 7, respectively.

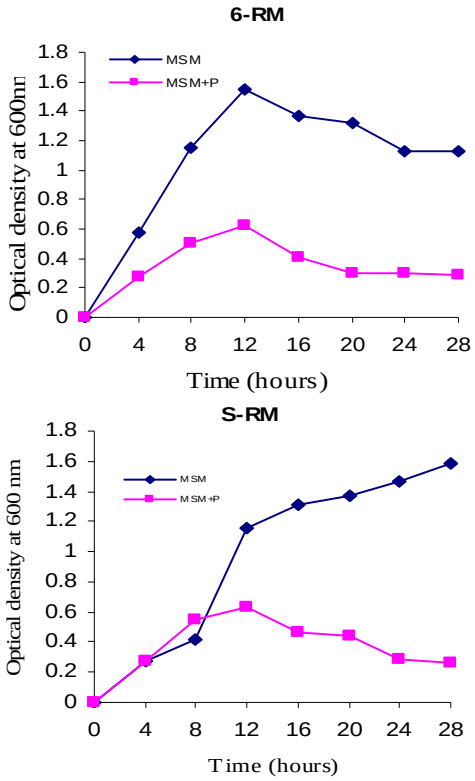


Fig. 1 Growth of bacterial isolates (6-RM, S-RM) in the presence of MS medium and MS medium containing phenol (100 µg/mL).

Besides analyzing bacteria with specific culture and biochemical methods, microbiologists rely on molecular tools particularly rDNA sequencing. The direct amplification of 16S rDNA gene enables one to identify the exact sample taken from the environment (Olsen *et al*., 1986). From the partial sequencing of 16S rRNA, the isolates used in this study were identified as *Stenotrophomonas maltophilia* (S-RM) and *Bacillus subtilis* (6-RM). The nucleotide sequences of their 16S rRNA gene segments were deposited in Genbank under accession numbers JQ396382.1 and JQ396383.1. The former is affiliated with class Gammaproteobacteria

while later belongs to Bacilli. Experiments by Whiteley and Bailey (2000) have demonstrated that organisms from class Gammaproteobacteria are dominantly prevalent in industrial phenolic waste system. They have also reported the presence of certain phenol degrading proteins among this group of bacteria. Previously, it has been shown that members of class Bacilli possess the ability of only degrading the aromatic hydrocarbons (Abd-El-Haleem *et al*., 2002). But the enzyme assays, protein estimation and HPLC have now shown that they are also capable of degrading phenol into some less toxic byproducts. Therefore, strain 6-RM can also be employed in bioremediation of phenol.

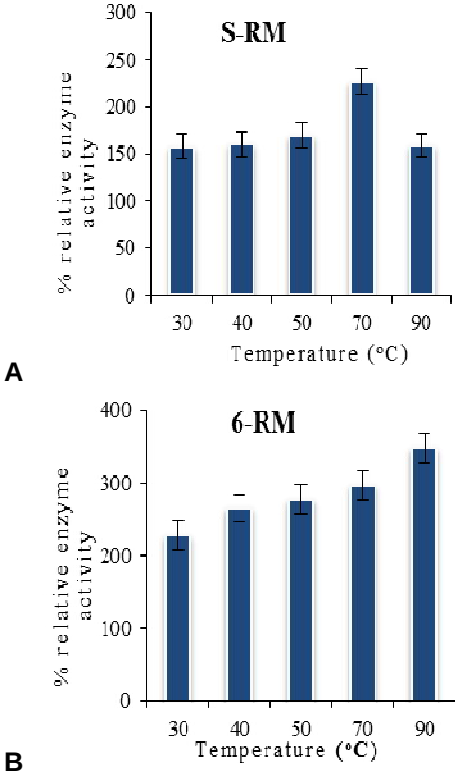


Fig. 2. The catechol-2,3-dioxygenase activity of S-RM (A) and 6-RM (B) was measured under assay conditions at different temperature.

Nature and characteristics of phenol degrading enzyme from S-RM and 6-RM

Microorganisms degrading phenol usually produce enzyme oxidoreductases which may cleave phenol at *ortho* or *meta* positions producing intermediates *cis,cis*-muconic acid and 2HMS. Each strain produces a single type of enzyme and it may also be possible that many different strains can produce similar type of enzyme. These enzymes (oxidases and peroxidases) are produced normally in the organism for their own needs and through excretion or lysis are released in the environment and mediate different activities (Sinsabaugh, 2010). The crude enzyme assays for both bacterial isolates S-RM and 6-RM showed maximum extracellular enzyme activity (350 and 290%) as compared to the intracellular (60 and 34%), respectively. Absence of absorbance at 260 nm indicated that both these isolates possess C23D enzyme. It also indicated that they may degrade phenol through meta-cleavage pathway (Banerjee and Ghoshal, 2010). Absorbance at 375 nm for strain S-RM slightly increased with increase in temperature and was highest at 90°C. Similarly, for isolate 6-RM enzyme activity increased in a linear fashion as the temperature was increased (Fig. 2). C23D enzyme present in local isolates S-RM and 6-RM appeared to be acidophilic protein as its activity decreased in alkaline pH (Fig. 3).

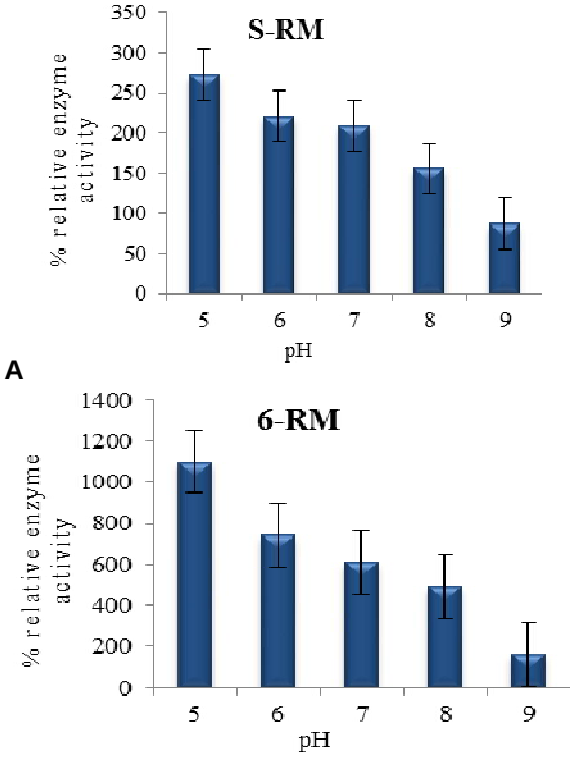


Fig. 3. The catechol-2,3-dioxygenase activity of S-RM (A) 6-RM (B) was measured under assay conditions at different pH.

Different metal ions are required as cofactors for maximum enzyme activity and at the same time some have inhibitory effect on the activity of enzyme. C23D is from the class of metalloproteins and generally uses iron

as a co factor but active site which uses Mn^{+2} as a cofactor is also known (Vaillancourt *et al*., 2006). Effect of metal ions on the enzyme activity is shown in figure 4. The enzyme activity of isolate 6-RM was inhibited in the presence of Zn^{2+}, Na^{1+} and Mn^{2+} while Cu^{2+} and Mg^{2+} enhanced the activity. For isolate S-RM only Zn^{2+} and Na^{1+} inhibited the enzyme activity while others ions had positive effect on the enzyme activity (Fig. 4).

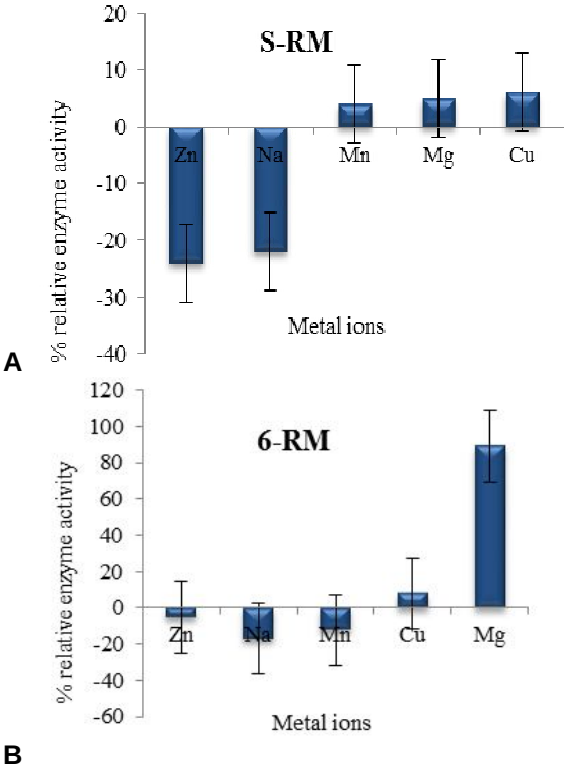
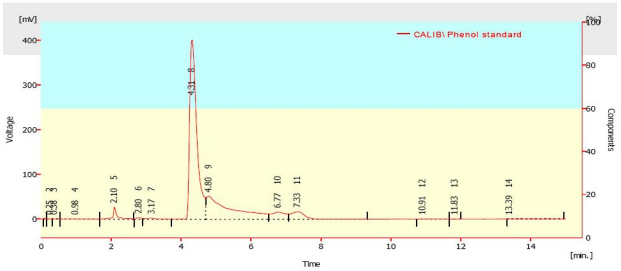


Fig. 4. Effect of metal ions on catechol-2,3-dioxygenase activity of S-RM (A) and 6-RM (B) bacterial strains.

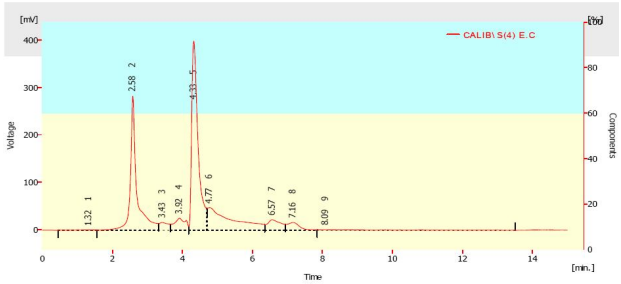
HPLC analysis

Data from HPLC (Fig. 5A-C) showed that phenol has been degraded by both isolates into its byproducts. From the enzyme assays it was concluded that phenol is degraded into 2HMS by the action of catechol-2,3-dioxygenase obtained from S-RM and 6-RM bacterial strains. The peaks obtained from HPLC have confirmed the production of 2HMS during degradation. It can also be inferred from the information that both isolates might be following meta-cleavage pathway for converting phenol into less toxic products (Kolvenbach *et al.*, 2014).

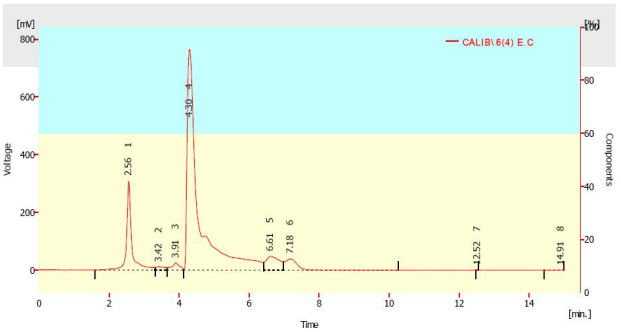
The purpose of this study was to isolate and characterize phenol degraders from the local environment. The experimental results showed that *S. maltophilia* and *B. subtilis* possess the ability to survive in the presence of phenol. As they were found able to survive due to the presence of phenol degrading enzyme C23D, which can convert phenol into 2HMS. Moreover, the activity of this enzyme was influenced by temperature, pH and metal ions. Absorbance spectra and HPLC were helpful to predict the cleavage pathway of phenol substrate. On the basis of above mentioned results, the investigated strains seem to possess prospects of their implication in wastewater treatment. These strains could be studied further for treatment of industrial effluent as well as natural terrestrial areas.



(A) Phenol standard



(B) S-RM



(C) 6-RM

Fig. 5. (A) HPLC chromatogram for phenol standard with a mobile phase of methanol and water (4:3) at mL/min and detection at 375 nm. HPLC of phenol treated with enzyme of isolate S-RM (B) and 6-RM (C) with same procedure as mentioned in the control.

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Conflict of interest

The authors have declared that no competing interests exist.

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