Characterization of Phenol Metabolizing Bacteria

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ORIGINAL ARTICLE

Isolation and Characterization of Phenol Metabolizing Bacillus cereus Strain SBBP4

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Abstract

US Environment Protection Agency has categorized phenol and its derivatives as persistent organic pollutants (POPs) due to the associated deleterious effects. Conventional mitigation approaches due to the complex procedures, high cost and involvement of lots of man power have failed in clearing this pollutant from ecosystem. Bioremediation based on bacteria can replace these conventional approaches due to simpler technology and low cost. In this study, isolation and characterization of phenol metabolizing bacteria from tannery industry soil was done. Isolation was carried out using minimal salt media (MSM) through serial dilution method. Followed this, bacterium was analyzed via growth curve analysis, ribotyping, biochemical testing, antibiotic sensitivity profiling, phenol removal assay and FTIR analysis. Bacterium was identified as Bacillus cereus. It was fast growing and capable of removing 98.7% phenol. It also exhibited resistance against amoxicillin, augmentin, cefadroxil, and cephalixin. Comparison of FTIR spectra of control vs bacterial sample also showed slight variation in peaks indicating the change in bonds of phenol in the presence of present study isolate. Bacterium Bacillus cereus strain SBBP4 can be investigated for further optimization and employed for efficient phenol removal.

1. INTRODUCTION

The colorless crystalline substance known as phenol has a distinct smell and is denoted by the molecular formula C₆H₅OH, which stands for carbolic acid. A hydroxyl (–OH) group and a benzene ring are closely bonded inside its structure (Galdino et al., 2023). Phenol is mostly produced by extracting tar from coal or by converting cumene, which is present in plants used to produce tar, into phenol (Gai et al., 2019). Alkylphenols, cresols, xylanols, resins, aniline, and different chemical processes are examples of industrial applications. It is also used in many different industries,
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including metallurgy, oil, insecticides, explosives, dyes, textiles, and chemical analysis as a reagent and disinfectant. Vehicle exhaust and atmospheric interactions introduce pollutants into the environment (Fini et al., 2022). Phenols have a variety of uses in both home and industrial settings (Mennani et al., 2022). It was first used as a surgical antiseptic by Joseph Lister in 1865, and it was successful in lowering the death rate during surgical procedures (Lachenmeier, 2014). Low-toxicity phenols, such as butylated hydroxytoluene (BHT) and n-hexylresorcinol, are used as antioxidants in food and in antiseptic compositions (Gucbilmez, 2022; Schmidt, 2023). Phenol is used in industry as a precursor for plastics, explosives, and medications like aspirin. Its derivatives are also used in wood preservation, photography, and dye manufacturing (González-Laredo et al., 2015). The primary factor influencing phenol toxicity is its reactivity with biomolecules within a cell, particularly its capacity to provide free electrons to oxidized substrates. Catalyzed by oxidative enzymes found in different organs such prostaglandins, myeloperoxidases, and peroxidases (de Oliveira et al., 2021). When phenol is consumed, it can cause central nervous system (CNS) abnormalities that can result in collapse and coma (Chae et al., 2020). Phenol exposure can irritate the eyes and in extreme situations, cause blindness. There are concerns that phenol exposure may be linked to genetic fiber defects, cancer, and paralysis (Said et al., 2021; Xu et al., 2021). Phenol damages the kidneys, liver, muscles, and eyes in addition to causing skin irritation and necrosis. When phenol reacts with the amino acids in collagen and epidermal keratin, it causes coagulation, which damages the skin. Phenol can be deadly at high doses; an adult may die from 1g. However, tolerance varies from person to person. Acute toxicity causes symptoms such as mucous membrane irritation, black urine, and dry mouth, whereas chronic exposure causes pathological alterations in multiple organs in animals (He et al., 2012).

When people are exposed to phenols, cytochrome P450 is microsomally activated, which is linked to the development of cancer. Some xenobiotics can be transformed into electrophilic forms that interact with biological structures through oxidation processes. Mice’s liver cancer development has also been connected to this process. Significantly, the degree of cytochrome P450 microsomal activity in hepatocytes is correlated with the development of cancer. Mice have the same metabolism of pentachlorophenols, but they do not develop cancer due to a far lower activation of this cytochrome (Huynh and Teel, 2002). After a variety of conventional approaches were tried, biodegradation was shown to be the most straightforward, economical, and successful way to reduce the amount of phenol in significant amounts of industrial effluent (Bibi et al., 2023; Eryılmaz and Genç, 2021). Algae, fungus, and bacteria are only a few of the microorganisms involved in biological degradation (Wu et al., 2022).

To lessen the negative consequences of phenol contamination, bioremediation techniques must be developed, which requires the isolation of phenol-degrading bacteria (Mohd, 2022). Keeping in view, these hazardous effects of phenol, present study has been designed in which we have isolated phenol remediating bacterium from tannery industry soil sample and characterized to get an insight into its phenol removal potential. This may result in the emergence of more effective and long-lasting methods for removing phenol from the environmental matrix and so lessening its harmful effects on ecosystems and public health.

2. METHODOLOGY

Isolation of phenol degrading bacteria

Firstly, we collected the soil sample from a tannery industry site located in Multan. Soil (1g) was incubated in Ringers soln. (20 ml) under shaking conditions for overnight at 37°C. Then serial dilutions of ringer’s soln. ranging from 10⁻¹ to 10⁻⁶ were prepared. Followed this, the dilutions were spread on petri-plates containing solidified minimal salt medium (MSM) enriched with phenol and incubated at 37°C for one week (Table 1). MSM used in present study was already reported in literature. Colony forming units, appeared on plate, were streaked to get purified colonies. The colonies obtained were then analyzed for morphological characteristics and gram staining.

Molecular characterization

An organic extraction technique was used to extract bacterial DNA. This approach involved use of lysozyme, Tris-
EDTA (TE) lysis buffer, SDS, proteinase K, chloroform: isoamyl alcohol, ethanol and isopropanol. Once washed and dried, the DNA pellet was kept in low TE buffer. DNA was quantified using agarose gel electrophoresis. Afterwards, 16S rRNA gene was amplified via forward AACMGGATTAGATACCGK and reverse primer GACGGGCGGTGWGTRCA. The amplicon size was 600 bp. Amplified PCR products were analyzed by resolving them on agarose gel and sent for Sanger sequencing to Macrogen, Korea. The FASTA files obtained were checked for top BLAST sequence homology using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi). After taxonomic identification of isolate, FASTA sequence was submitted to NCBI GenBank database and accession number was assigned.

Phylogenetic tree was constructed using Phylogeny.fr (Available at www.phylogeny.fr/advanced.cgi). The workflow designed to construct tree comprised of multiple alignment by MUSCLE, alignment curation by Gblocks, construction of phylogenetic tree by PhyML and visualization of phylogenetic tree by TreeDyn. Bootstrapping method with bootstrap value of 100 was selected.

**Growth curve analysis**

After preparing a fresh MSM broth medium (5 ml), the overgrown bacterial culture (100 µl) was added to it as inoculum. After that, the cultures were kept in a shaking incubator at 37°C at 150 rpm. The absorbance (OD600) was measured at 600 nm spectrophotometrically to determine the bacterial cells growth. The OD600 was estimated at different time intervals i.e. 0, 3, 6, 9, 24, 27, 30, 48, 51, 54, 68, 71 and 74 hr. Experiment was performed in triplicates using synchronized cultures. Growth curves were created by plotting the OD600 values vs time in order to comprehend the growth pattern.

**Biochemical characterization of bacteria using RapID STR System**

Using the Remel RapID™ ONE panel System, biochemical characterization of bacteria was carried out. This involved inoculating panels with bacterial cultures that had grown over night and interpreting 19 test findings from 18 reaction cavities. For some cavities, the bifunctional testing method was used. After incubation and reagent injection, findings were recorded in accordance with the provided interpretation guide. The tests performed included urea hydrolysis test (URE), arginine dehydrolase (ADH), ornithine hydrolysis test (ODC), lysine hydrolysis test (LDC), aliphatic thiol utilization test (TET), fatty acid ester hydrolysis test (LIP), sugar aldehyde utilization test (KSF), sorbitol fermentation test (SBL), GUR, ONPG, βGLU, βXYL, p-nitrophenyl-n-acetyl-β, Dglucosaminide (NAG), malonate utilization test (MAL), proline naphthylamide test (PRO), γ-glutamyl-β-naphthylamide test (GGT), pyrrolidine β-naphthylamide test (PYR), adonitol utilization test (ADON) and tryptophane utilization test (IND).
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**Antibiotic sensitivity profiling**

Amoxicillin, augmentin, cefadroxil, and cephalaxin were used to assess the antibiotic resistance potential in isolated bacteria. After filter paper discs were soaked in calculated antibiotic concentrations and put on nutrient agar plates, the plates were incubated for 24 hr at 37˚C. After this, the inhibitory zone widths of isolate against each antibiotic were determined.

**Phenol removal estimation**

The assay was performed in triplicates using synchronized cultures. To get the synchronized cultures, overnight grown bacterial culture (100 µl) was inoculated into the phenol-supplemented MSM broth. Phenol concentration was determined by measuring the absorbance (OD270) of supernatant at 270 nm following incubation and centrifugation at 0 hr and at the end of log phase. Percent phenol removal was estimated by following formula.

\[
\text{Percent phenol removal (% age)} = \frac{(\text{initial OD}_{270} - \text{final OD}_{270})}{\text{initial OD}_{270}} \times 100
\]

**FTIR analysis**

To further confirm the phenol degradation potential of bacterium, FTIR analysis was carried out. For this task, phenol supplemented MSM medium (5 ml) was inoculated with 100µl of overnight grown bacterial suspension. Tubes were kept in shaking incubator till the logarithmic growth was achieved. When the culture reached the log phase, tubes were centrifuged. Supernatant was sent for FTIR analysis to determine the changes in phenol in the presence of bacteria. The change in peaks was confirmed through comparison with control comprising of the MSM and phenol.

3. **RESULTS**

**Colony Forming Units (CFUs)**

The colony of phenol-metabolizing bacteria documented in present study, was obtained in plate inoculated with $10^{-1}$ dilution. The colony was initially marked as C4. The CFUs for this isolate were calculated to be $35 \times 10^{-1}$ (Figure 1). The bacterium was found to be Gram positive.

![Figure 1: Isolation of phenol metabolizing bacteria from tannery industry soil using serial dilution method. Bacterial colonies obtained at (a) control (b) $10^{-1}$ dilution.](image)

**Molecular Characterization**

DNA was extracted of bacterial strain with help of bacterial colony and it was amplified and products were observed on gel (Figure 2).
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The bacterium was identified to be Bacillus cereus. Strain assigned to this bacterium is SBBP4. SBB stands for School of Biochemistry and Biotechnology where the research work was carried out and P stands for phenol (Table 2). Phylogeny analysis revealed the relatedness of B. cereus SBBP4 with B. cereus strain BHW-98 (Figure 3). As both were originating from same branch point. These two isolates were also sharing clade with B. subtilis showing their closeness with this bacterium. However, B. cereus strain SBBP4 was distantly related with Brevibacillus brevis and Acinetobacter lwofii strain DSM 2403.

![Figure 2: Agarose gel electrophoresis results for phenol degrading bacterial (a) extracted DNA and (b) amplicon.]

![Figure 3: Construction of phylogenetic tree using phylogeny.]

Table 2: Top BLAST sequence homology, % age similarity and accession number assigned to phenol degrading bacterium.

<table>
<thead>
<tr>
<th>Top BLAST homology</th>
<th>% age similarity</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus cereus</td>
<td>100</td>
<td>OR272109</td>
</tr>
</tbody>
</table>

**Growth curve analysis**

Different growth phases i.e. lag, log, stationary and death phases were identified with reference to the OD600 values measured at different time intervals. Lag phase was observed ranging from 0 to 27 hr whereas log phase started after 27 hr and lasted at 48 hr. Stationary phase ranged between 49 to 54 hr and at 55 hr death phase started (Figure 4).

**Biochemical characterization**

The B. cereus SBBP4 showed positive result for On subject to biochemical characterization our bacterial strain showed positive results for ADH, ODC, LDC, ONPG, βGLU, NAG, MAL and PYR tests (Figure 5).
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**Figure 4:** Growth curve analysis of *B. cereus* SBBP4.

**Figure 5:** Results of biochemical characterization of *B. cereus* strain SBBP4 performed using RapIDTM ONE panel System.

**Antibiotic sensitivity profiling**

The antibiotic sensitivity profiling of *B. cereus* SBBP4 revealed resistance potential against amoxicillin (5 µg) and azithromycin. In rest of the cases, sensitivity was observed as zones of inhibition were formed. Maximum zone was observed in case of augmentin and amoxicillin at conc. of 15 µg and in cefadroxil at 10 µg.

**Phenol removal efficiency estimation**

Phenol removal efficiency of *B. cereus* SBBP4 was estimated via measuring the residual phenol in supernatant. Values of OD270 were found to be 3.0 ± 0 and 0.038 ± 0.0007 at 0 and 49 hr, respectively. Degradation efficiency determined to be 98.7% / 49 hr.

**FTIR analysis**

Comparing the FTIR spectra of *B. cereus* SBBP4 with a control, revealed small alterations in peak patterns indicating possible modifications to the bond's bending and stretching (Figure 6).
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![Image](a)

![Image](b)

**Figure 6**: FTIR spectra for confirmation of changes in phenol structure induced by *B. cereus* SBBP4 (a) Control (b) *B. cereus* strain SBBP4.

### 4. DISCUSSION

Present study involved the isolation and characterization of *B. cereus* SBBP4. The phenol degradation ability of bacterium was confirmed using MSM, phenol removal assay and FTIR analysis. *B. cereus* SBBP4 is found gram positive but many gram negative phenol degrading bacteria have also been isolated (Van Schie and Young, 1998). Present bacteria was found to belong to genus *Bacillus* and species *cereus*. Literature contains data reporting *B. cereus* as phenol degrading bacteria (Banerjee and Ghoshal, 2011; Singh et al., 2009; Tahya et al., 2019). Hence, our findings are in accordance with previous reports on phenol metabolizing bacteria. Bacteria capable of using phenol as carbon source and belonging to different genera and species have also been reported like *Bacillus thurinfgiensis*, *Pseudomonas humanensis*, *Pseudomonas putida* and *Arthrobacter globiformis* (Ereqat et al., 2018; Wang et al., 2021).

The biochemical tests i.e. TET, LIP, KSF, SBL, GUR, ONPG, βGLU, βXYL, NAG, MAL, PRO, GGT, PYR, ADON and IND, performed for *B. cereus* SBBP4 have never been conducted for any phenol remediating bacteria. However, some other methods like API 20 NE system, BioMerieux SA identification kit has been used for biochemical characterization. In addition to this, some other tests like catalase production test, maltose, glucose, mannose, lactose, sucrose, mannitol, potassium gluconate and N-acetyl glucosamine tests have also been performed. Finding of positive result for ADH, ODC and LDC is consistent with literature (Paisio et al., 2012).
Phenol degrading efficiency has been estimated by measuring residual phenol in supernatant. i.e. 98.76% / 49 hr. Literature reports removal efficiency of 88.6% / 96 hr in *Bacillus thuringiensis* and 95% in *Halomonas* sp. strain PH2-2 (Ereqat et al., 2018; Haddadi and Shavandi, 2013). Hence, *B. cereus* SBBP4 removal efficiency is comparable with the one reported in literature. Comparison of FTIR spectra of control versus bacterial sample has supported the bending and stretching in phenolic bonds, breakdown of existing bonds and formation of new one in bacterial sample. Although metabolomics approaches like GC-MS and LC-MS have been targeted for confirmation of phenol use as carbon source. Yet no one has confirmed the phenol degradation potential of bacteria via FTIR analysis. So, this is the first ever study using this approach in this regard.

5. CONCLUSION

Findings of present study confirmed use of phenol as carbon source by present study bacteria. Degradation potential of *B. cereus* SBBP4 can be further confirmed through metabolites identification via gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS) as well as at genetic level. The genes of bacterium can then be isolated and cloned in non-virulent system for green bioremediation of phenol-based pollutants.

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Author contributions

S.M.B performed analysis, F.M designed methodology, M.K write up of manuscript, N.H bioinformatics

Competing interests

The authors have no competing interests.

Data availability statement

The sequence of 16S RNA gene of *B. cereus* SBBP4 is available with accession ID OR272109 on NCBI GenBank database.

Submission declaration and verification

The work is not published previously and it is not under consideration for publication elsewhere.

6. REFERENCES

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