



# Molecular Identification and Characterization of *Bacillus* sp. NIGAB-1 for Phenol Degradation Under Saline Conditions

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**Background:** Phenol is an aromatic pollutant in industrial wastes that in combination with salts is highly toxic for all forms of life. Phenol elimination is the foremost challenge to meet the goal of pollutant-free environment.

**Objective:** The present study was carried out to isolate phenol degrading bacteria which can degrade phenol under saline conditions and to identify the isolated strains using 16S rRNA gene sequence analysis.

**Material and Methods:** Sediment samples were collected from Rawal Lake, Islamabad, Pakistan and enriched in mineral salt medium (MSM) containing phenol (150 mg.L<sup>-1</sup>). Isolated strains were identified on the basis of 16S rRNA gene sequence analysis. Growth of strains were tested at different pH, NaCl concentrations and temperature using Tryptic Soy Agar (TSA). Tolerance to phenol (0-750 mg.L<sup>-1</sup>) was checked at 5% NaCl and phenol degrading experiment was performed at 4% NaCl, pH 7 and 30 °C. In both, phenol tolerance and degradation study, phenol was used a sole source of carbon and energy.

**Results:** Thirteen bacterial strains were isolated after enrichment among which, NIGAB-1 was found capable of degrading phenol in saline conditions. This strain was identified as *Bacillus* sp. NIGAB-1 on the basis of 16S rRNA gene sequence analysis and the closest match was *Bacillus marisflavi* with 99.71% sequence identity. The *Bacillus* sp. NIGAB-1 exhibited best growth at 30 °C at pH 7 with 10% NaCl. The optimum phenol concentration for growth was recorded as 300 mg.L<sup>-1</sup>. This strain degraded 300 mg.L<sup>-1</sup> of phenol at 4% NaCl in 120 hours with the average degradation rate of 2.63 mg.L<sup>-1</sup>.h.

**Conclusion:** These findings suggest that this strain could be efficient in phenol degradation at adverse environmental conditions and helpful in remediation of phenol where the salt concentration is high.

**Keywords:** 16S rRNA gene; *Bacillus*; Biodegradation; HPLC; Phenol; Sludge.

## 1. Background

Phenol is an environmental pollutant which is widely distributed in waste of various industries like varnish, steel plants, textile, leather, resin manufacturing and dye industry (1, 2). Phenol has high solubility in water, hence accumulated in water used for drinking and agriculture purposes (3). Even in low concentration, phenol can harm living form, therefore, Environmental Protection Agency (EPA), USA listed phenol as a priority pollutant (4).

A voluminous body of research unveils phenol toxicity on microorganisms, plants, fish, and animals (5). Presence of phenol in water, even at low concentration can cause toxicity to aquatic life and unpleasant smell (6). Naresh et al. (7) reported disorders of central nervous system, hypothermia, whitening of the cornea and occasionally blindness, hepatic damage etc. in human due to skin contact with phenol. Phenol has also

been reported as phytotoxic. It is a potential inhibitor of plant growth and germination in rice, reduces chlorophyll contents and negatively affects plants roots and seeds (8). In a report by Feng et al. (9) phenol adversely effected roots and seeds of Chinese cabbages resulting in yield losses. Due to harmful and deleterious nature of phenol, its elimination from environment is inevitable. Hexacarbon ring of phenol confers resistant to degradation by natural process however, the OH group make the degradation process a bit easier.

Many physiochemical method such as adsorption, coagulation, ion exchange, chemical oxidation, and photocatalytic oxidation methods are practiced to eliminate phenol from environment but microbial degradation is gaining more attention because of low cost and environment-friendly nature (10). Phenol degrading microorganisms include Algae (11), yeast (3) and

bacteria (12). Among microorganisms bacterial strains are extensively studied, and a lot of bacterial genera are reported to be involved in phenol degradation. These genera comprise *Rhodococcus*, *Stenotrophomonas*, *Lysinibacillus*, *Comamonas*, *Microbacterium* and *Pseudomonas* etc. (13). Due to higher utilization of salt in industries, effluent became saline, and phenol degradation in saline environment is difficult for those microorganisms which are not adapted to salinity (14). However, Halotolerant/halophilic microbes can withstand saline environment and continue their activities. On the basis of 16S rRNA sequencing we were able to identify *Bacillus* sp. NIGAB-1, a close match *Bacillus marisflavi* (AF483624), which can degrade 300 mg.L<sup>-1</sup> of phenol at 4% NaCl in 120 hours with the average degradation rate of 2.63 mg.L<sup>-1</sup>.h.

In Pakistan, few reports are available on biodegradation of phenol (15, 16). Overall, the degradation of phenol in saline condition remains a grossly neglected topic.

## 2. Objective

Keeping this gap in view, we embarked on isolation and characterization of bacterial strains capable of degrading

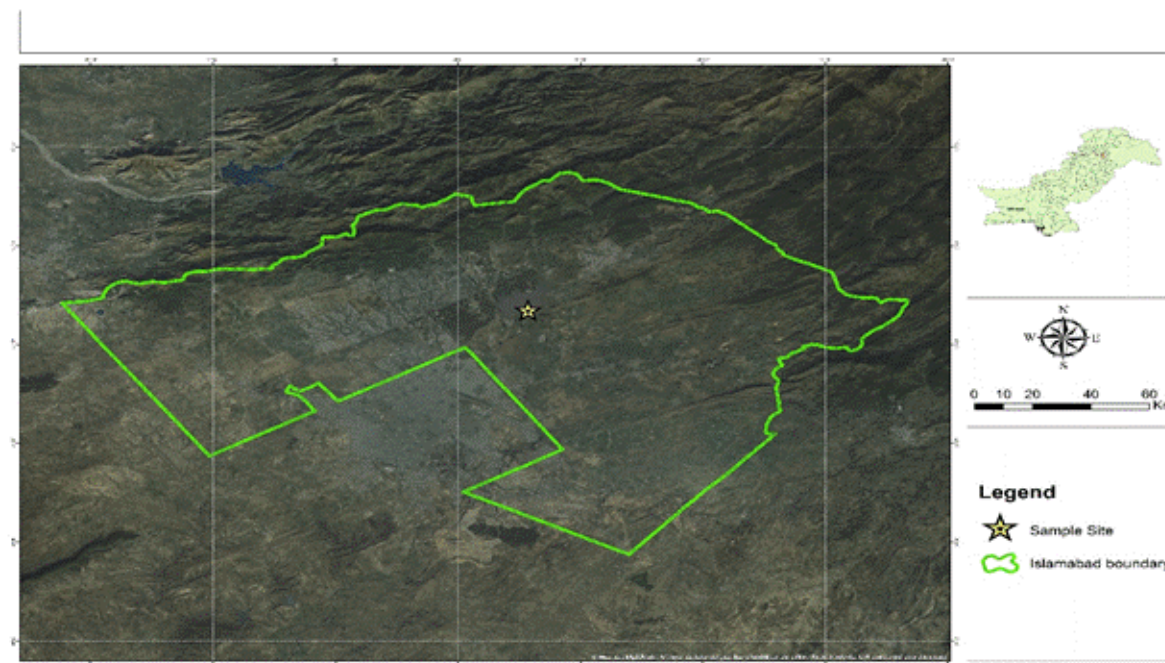
phenol in saline conditions and its identification on the basis of 16S rRNA gene sequence analysis.

## 3. Materials and Methods

This research work was carried out at National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Centre, Islamabad, Pakistan. Chemicals used in this research were purchased from SIGMA-ALDRICH (Germany). Phenol used in this study was of analytical grade.

### 3.1 Isolation and Enrichment

Sediment samples were collected from Rawal Lake, Islamabad, Pakistan (**Fig. 1**). Thirty ml sludge sample was enriched in Mineral Salt Medium (MSM) containing 5% NaCl and 150 mg. L<sup>-1</sup> phenol as a sole source of carbon and energy in a flask, and placed on shaker for six days. After six days, 50 µL of samples were spread on MSM plates containing 200 mg.L<sup>-1</sup> phenol with 5% NaCl and incubated at 28 °C till growth. Morphologically different colonies were purified on MSM plates containing phenol (200 mg. L<sup>-1</sup>). Strains were preserved in 70% glycerol and kept at -80 °C.



**Fig 1.** Map showing the location where samples were collected. Legend is also given.

### 3.2 Identification and Phylogenetic Analysis

Identification of bacterial strain was attempted through sequence analysis of 16S rRNA gene. Single colony of each strain was homogenized in TE buffer in PCR tube and placed in thermal cycler at 95 °C for 10 minutes. Sample was centrifuged and supernatant was collected and used as DNA template. Universal primers 9F and

1510R were used for amplification of 16S rRNA gene according to Ahmad *et al.* (17). Amplified products were confirmed through gel electrophoresis (1%) and purified using commercially available DNA purification kit (Invitrogen). Purified products were sequenced using internal primers 27F and 1492R. The 16S rRNA gene sequences obtained were refined using BioEdit

software to get the consensus sequence for each strain. The assembled consensus sequences were Blast (Basic Local Alignment Search Tool) searched using Ez-Taxon Server (<https://www.ezbiocloud.net/>) to retrieve sequences of 16S rRNA gene of closely related validly published species for exact identification of the isolated strains Ahmad *et al.* (17). On the basis of maximum identity score, sequences were selected and alignment was generated using Clustal W (version 1.6) (18). Phylogenetic tree was constructed using Neighbor-Joining algorithm contained in MEGA version 5 software package (19).

### 3.3 Biochemical Characterization

Consumption of different carbon sources by the isolated strain was determined using API 20E kit (bioMerieux, France). Few pure colonies (16 to 18 h old culture) of isolated stain were added in 0.85% saline solution and the microtubes of API 20E kit were filled with prepared inoculums. The kits were then placed in incubator at 28 °C for 24-48 h. The results were recorded according to color change.

### 3.4 Growth Conditions Optimization of Isolated Strain

The bacterial strain was tested for growth at different pH (4, 5, 6, 7, 8, 9, 10 and 11), temperature (4, 10, 20, 28, 35, 37, 40, 45, and 50 °C) and NaCl concentration (0-40%) in Tryptic Soy Broth (TSB). Growth was determined in terms of optical density (OD) at 600 nm using spectrophotometer (IMPLEN, Germany).

### 3.5 Phenol Tolerance and Degradation of Isolated Strain

Isolated bacterial strain was screened on MSM containing different concentration of phenol and 5% NaCl. Pre-culture was first prepared by growing strain on MSM broth supplemented with 100 mg.L<sup>-1</sup> phenol as a sole source of energy and carbon. This pre-culture was used to inoculate MSM broth containing 0, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700 and 750 mg.L<sup>-1</sup> phenol with 5% NaCl. MSM broth containing corresponding phenol concentrations without inoculation was used as blank. Growth was determined by taking optical density readings at 600 nm.

Range and optimum phenol concentration was determined for growth of the strain. Optimum phenol concentration was used for degradation potential at different NaCl concentrations. All these experiments were performed in triplicates. Residual phenol was measured through High Performance Liquid Chromatography (HPLC) according to conditions used by Ahmad *et al.* (13). Identification of phenol was inferred on the basis of retention time, and quantification

of residual phenol was done on the basis of six point external standard calibration curve.

### 3.6 Statistical Analysis

The data obtained from phenol degradation experiment by isolated strain were subjected to regression analysis using SAS 9.2.

## 4. Results

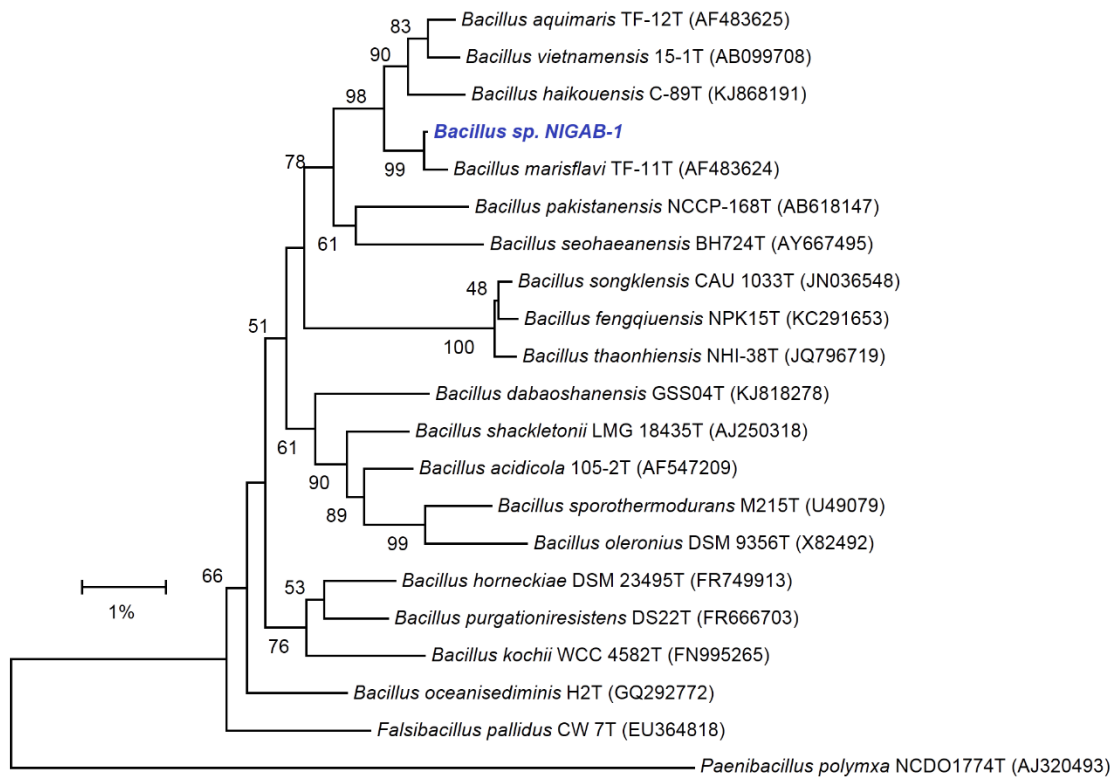
### 4.1 Isolation and Identification of New Bacterial Strain

The purpose of this study was to explore bacterial strains capable of degrading phenol in saline conditions. Thirteen morphologically distinct bacterial strains were isolated after enrichment (20). Among these strains, the fast growing strain was isolated, and designated as *Bacillus* sp. NIGAB-1 after molecular identification. The newly identified strain *Bacillus* sp. NIGAB-1 was further screened and evaluated for utilization of various carbohydrates, phenol tolerance and phenol degradation potential in saline conditions.

The sequence obtained was BLAST searched on Ez-taxon server to find out the closest match. The blastn revealed that NIGAB-1 share 99.71%, 98.57%, and 98.47% sequence identity (1372 bp) with *B. marisflavi* (AF483624), *B. oryzaecorticis* (KF548480) and *B. aquimaris* (AF483625), respectively. Phylogenetic analysis confirmed the affiliation of this strain with *B. marisflavi* (AF483624) with 99% bootstrap value (**Fig. 2**).

### 4.2 Physiological and Biochemical Characterization

Growth of *Bacillus* sp. NIGAB-1 was tested at different pH, NaCl concentrations and temperature variations. *Bacillus* sp. NIGAB-1 exhibited maximum growth at pH 5-9 whereas, optimum pH was recorded as 7. Though strain could grow in the range of 10-45 °C but the optimum temperature was 30 °C (**Table 1**). Similarly, NIGAB-1 showed maximum growth at 8-10.0% NaCl indicating NaCl tolerance. Nevertheless, this strain was able to grow at 0.0-24% NaCl. **Table 2** demonstrates the biochemical characterization of selected strain. After 48 hours of incubation, the strain *Bacillus* sp. NIGAB-1 showed positive results for β-galactosidase, Tryptophan deaminase, Arginine dihydrolase, Citrate utilization, Gelatinase, sodium pyruvate, NO<sub>2</sub> production fermentation/oxidation of Glucose, Mannitol, Melibiose, Rhamnose and Sorbitol while rest of the substrates tested including H<sub>2</sub>S production, Indole production and Lysine decarboxylase did not showed any activity.



**Fig 2.** Phylogenetic tree showing relationships strain NIGAB-1 with the most closely related species inferred from sequences of 16S rRNA gene. *Paenibacillus polymxa* (AJ320493) was used as an out-group. The tree was generated using the Neighbor-Joining method. Bootstrap values are 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

**Table 1.** Optimization of conditions for growth of *Bacillus* sp. NIGAB-1

Strain	NaCl (%)		Temperature oC		pH	
	Optimum	Range	Optimum	Range	Optimum	Range
<i>Bacillus</i> sp. NIGAB-1	10,	0.0-24.0	30	10.0-45.0	7	5.0-9.0

**Table 2.** Biochemical characteristics of *Bacillus* sp. NIGAB-1 using API 20E kit Table 1

Biochemical tests	<i>Bacillus</i> sp. NIGAB-1	Biochemical Tests	<i>Bacillus</i> sp. NIGAB-1
$\beta$ -galactosidase	+	<i>Fermentation/oxidation of:</i>	
Arginine dihydrolase	+	Amygdalin	-
Citrate utilization	+	Arabinose	-
Gelatinase	+	Glucose	+
H <sub>2</sub> S production	-	Inositol	-
Indole production	-	Mannitol	+
Lysine decarboxylase	-	Melibiose	+
Ornithine decarboxylase	-	Rhamnose	+
Sodium pyruvate	+	Saccharose	-
Tryptophan deaminase	+	Sorbitol	+
Urease	-	Nitrate reduction	+



### 4.3 Phenol Tolerance and Degradation

In order to test the phenol tolerance *Bacillus* sp. NIGAB-1 was evaluated for its growth on different concentrations of phenol at 5% NaCl. The selected strain could grow at 50-550 mg.L<sup>-1</sup> of phenol while negligible or no growth was observed at 600-750 mg.L<sup>-1</sup> phenol. Optimum phenol concentration for NIGAB-1 was recorded as 300 mg.L<sup>-1</sup> where optical density (OD<sub>600nm</sub>) reached to 0.405. However, nearly same growth was observed at 350 mg.L<sup>-1</sup> phenol (Fig. 3).

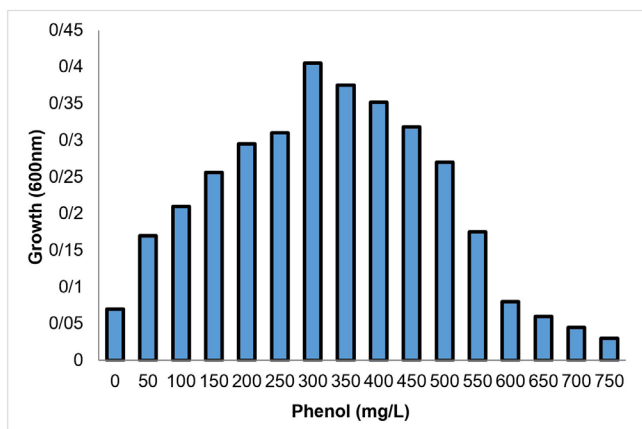


Fig 3. Tolerance of NIGAB-1 at different concentrations of phenol in MSM supplemented with 5% NaCl.

For determination of phenol degrading potential, selected strain of *Bacillus* sp. NIGAB-1 was inoculated in MSM broth containing 50 mg.L<sup>-1</sup> phenol and 5% NaCl. In all experiments performed, phenol was used as a sole source of carbon and energy. After two days, the culture was observed as turbid and used as an inoculant for MSM broth containing 300 mg.L<sup>-1</sup> phenol and 10% NaCl. But, at this concentration, no growth of bacteria was observed (data not shown). NaCl concentration was then reduced to 8%, 6%, and 4%, and phenol concentration was kept same. No growth observed at 8% NaCl, and negligible growth was recorded at 6% NaCl in the presence of 300 mg.L<sup>-1</sup> phenol (data not shown). However, at 4% NaCl, tremendous growth was observed. Phenol degrading experiment was performed in triplicate. In each flask, 300 mg.L<sup>-1</sup> phenol was added to 50 ml of MSM broth and placed on shaker at 28 °C. Two types of controls were run in parallel. One control was MSM broth with inoculum and without phenol, and second control was MSM broth containing 300 mg.L<sup>-1</sup> phenol with no inoculum. At 12 hours interval, one ml sample was taken and checked for growth by the use of spectrophotometer. Results showed a 24 hours lag phase with very low optical density (OD). Log phase was started from 24 hours and continued to 84 hours.

Stationary phase was recorded for only 12 hours which was continued from 84 to 96 hours of inoculation, and after that decline phase was started. Maximum OD stain was incubated for a total period of 120 hours. No growth was observed in controls. One ml sample from culture was taken and used to measure the residual phenol. For this purpose, six point external standards (50, 100, 150, 300, 500, 800 mg.L<sup>-1</sup>) of phenol were prepared and analyzed on HPLC (Fig. 4).

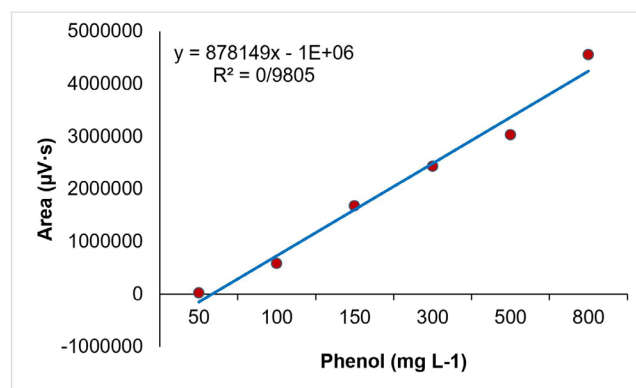


Fig 4. Peak area obtained from six points external standards.

Remarkably, *Bacillus* sp. NIGAB-1 completely degraded 300 mg.L<sup>-1</sup> phenol in 108 hours with the average degrading rate of 2.63 mg.L<sup>-1</sup>.h. Degradation rate was not constant and reached to peak at 24 hours (Fig. 5). At time zero, no phenol degradation was recorded. After 12 hours of incubation, 12.5% phenol degradation was observed where the OD reached to 1.1. Highest growth was recorded at 96 hours of incubation (OD 0.47) where 96.26% phenol degradation was observed.

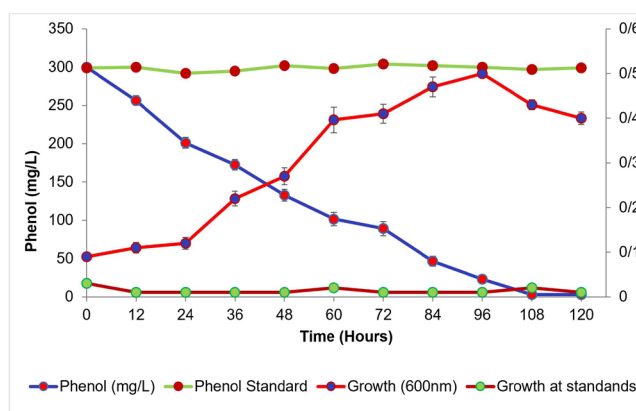


Fig 5. Phenol degradation by *Bacillus* sp. NIGAB-1 in MSM broth containing 4% NaCl and 300 mg.L<sup>-1</sup> phenol.

The data obtained from *Bacillus* sp. NIGAB-1 growth in presence of phenol with respect to time was analyzed statistically. The correlation value of growth with

respect to time of incubation was observed as 0.9 which showed strong degree of relationship in positive direction, and negative correlation was found between phenol degradation with time of incubation (-0.98) and growth of *Bacillus* sp. NIGAB-1 with phenol degradation (-0.93). Regression analysis was performed for *Bacillus* sp. NIGAB-1 growth with respect to time which revealed the significance of F value (81.3). From this value, we can conclude a significant difference in growth of bacteria in phenol containing medium with respect to time. However, F value was recorded as 277.63 for phenol degradation with respect to time which is greater than that for bacterial growth with time of incubation. Coefficient of variance for bacterial

growth with respect to time and phenol degradation with respect to time was recorded as 13.43 and 15.74, respectively, which are within range <20. Coefficient of determination  $R^2$  and Adjusted  $R^2$  value for growth of *Bacillus* sp. NIGAB-1 was calculated as 0.90 and 0.89 respectively, and the closeness between these two values indicates the accuracy of the model. Similarly,  $R^2 = 0.97$  and Adjusted  $R^2 = 0.96$  were calculated for phenol degradation with respect to time. P value for the growth of *Bacillus* sp. NIGAB-1 in terms of time and phenol degradation with respect to time was calculated as 0.0001, which is smaller than 0.05 indicating that the model terms are significant (**Table 3**).

**Table 3.** Analyses of Variance (ANOVA) for Bacterial growth and phenol degradation with respect to time

<b>Bacterial Growth With Respect to Time</b>					
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.17361	0.17361	81.23	<.0001
Error	9	0.01924	0.00214		
Corrected Total	10	0.19284			
<b>Phenol Biodegradation With Respect to Time</b>					
Model	1	100602	100602	277.63	<.0001
Error	9	3261.28764	362.36529		
Corrected Total	10	103864			

## 5. Discussion

The role of halophilic bacteria in phenol degradation can hardly be exaggerated. In the present study we embarked on isolation and characterization of bacterial strains capable of degrading phenol in saline conditions. On the basis of 16S rRNA sequencing, we were able to identify *Bacillus* sp. NIGAB-1, a close match *B. marisflavi* (AF483624), which can degrade 300 mg.L<sup>-1</sup> of phenol at 4% NaCl in 120 hours with the average degradation rate of 2.63 mg.L<sup>-1</sup>.h.

Based on 16S rRNA gene sequence analyses, NIGAB-1 was identified as the member of the genus *Bacillus* which shares 99.71% sequence identity with *B. marisflavi* (AF483624). Optimum pH, NaCl and temperature of the isolated strain were recorded as 7, 10%, and 30°C, respectively. This strain was evaluated for phenol degradation at various initial phenol and NaCl concentrations, and its optimum efficiency was observed as degradation of 300 mg.L<sup>-1</sup> phenol in 108 hours. This strain is useful in degradation of wastewater containing phenol where salt concentration is high. The strain identification merely on morphological bases is not satisfactory. It may give false positive results as environmental factors like pH, temperature and composition of media etc. affect bacterial colony

morphology. In bacterial identification and phylogenetic analysis, 16S rRNA gene is convenient at genus level identification. This gene plays a crucial role in protein translation and being housekeeping gene not lost from genome. The sequence length of 16S rRNA gene is enough to get information regarding evaluation and subsequently the taxonomic position of a bacterium (21). These results are in line with a little variation with that of Yoon *et al.* (22) where they isolated *B. marisflavi* (AF483624) from seawater and validated as novel bacterial strain. *B. marisflavi* grew well at pH 6-8, temperature 30-37°C and NaCl 0-5%.

We compared the biochemical results with closest match of our strain (*B. marisflavi*), which was first identified as novel strain by Yoon *et al.* (22). Results for Urease, Glucose, and Inositol are same while observations for Rhamnose, Saccharose, and Sorbitol show contradiction.

A lot of research reports unveiled the inhibition of bacterial growth at higher concentration of phenol. Bacterial growth inhibition at high phenol concentration is considered as substrate inhibition phenomenon (23). Phenol tolerance and phenol degrading potential of bacteria is considered not to be interlinked. Various phenomena may be involved towards this property.

This may be due to horizontal gene transfer and mutations in gene carrying phenol degrading character (24). Bacterial potential to withstand different level of phenol has great impact because phenol concentration in industrial waste fluctuates (25).

Members of *Bacillus* are Gram-positive in nature. Species of this genus are reported for showing tolerance to different pollutants including organic compounds. This property is the result of structure and permeability of cell membrane, production of surface active agent and enzymes involved in degradation process (26). The closest match of *Bacillus* sp. NIGAB-1 is *B. marisflavi* (AF483624), which is reported neither for phenol degradation nor phenol degradation in saline condition. This the first report on evaluation of *B. marisflavi* for phenol tolerance and phenol degradation in saline condition. The potential of phenol degradation by *B. marisflavi* is yet to be studied, and not a single report is available. Nevertheless, in a study conducted by Nadaf and Kanase (27) degradation of Congo red and methyl blue by *Bacillus marisflavi* is reported. However, there are many reports available on phenol degradation by other members of *Bacillus*. Banerjee and Ghoshal (28) characterized bacterial strain AKG1 for phenol degradation which belonged to genus *Bacillus* and had 99.63% sequence identity with *B. cereus*. For this strain, 600 mg.L<sup>-1</sup> phenol was found optimum for growth. It is not necessary for a bacterium to have the potential to degrade the same amount of phenol to which it is tolerant. Zhou *et al.* (29) reported the isolation of *Sulfobacillus acidophilus* TPY which had the ability to degrade phenol. To determine optimum phenol tolerant level of this bacterium, various phenol concentrations were used. Strain showed tolerance to some level but with the increase in phenol concentration, growth was reduced and completely inhibited at 1500 mg.L<sup>-1</sup> phenol. This strain was able to degrade only 100 mg.L<sup>-1</sup> of phenol.

There are many reports which highlight the biodegradation property of members of the genus *Bacillus*. For instance, Feitkenhauer *et al.* (30) reported the isolation of *B. themoleovorans* sp. A2 which was efficient in phenol degradation in saline condition. Another study conducted by Gayathri and Vasudevan (31) explored phenol degrading potential of bacterial consortium isolated from phenol contaminated site which was saline. It was found that this consortium can efficiently degraded 50 mg.L<sup>-1</sup> phenol in four hours in the presence of 10% NaCl. The consortium was composed of three *Bacillus* members.

Taken together, the newly identified *Bacillus* sp.

NIGAB-1 has great potential for phenol degradation under saline conditions. This study generates very valuable information regarding biological treatment of saline wastewater containing phenol.

### Conflict of interests

The authors have no conflicting interests, and all authors have approved the manuscript and agree with submission of its final version.

### Authors' Contribution

All authors of this research paper have directly participated in the planning, execution, or analysis of this study.

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