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Full Length Article

Evaluation of a Locally Isolated Phenol Degrading Bacterium under different Physiological Conditions

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Abstract

Phenol is an organic pollutant accumulated into the environment either naturally or by anthropogenic activity which adversely affects all living forms regarding mortality, health, quality and production. The elimination of phenol is obligatory to meet the goal of the untainted environment and sustainable agriculture. Bioremediation plays a vital role and preferred over physiochemical methods owing to its environment-friendly nature and low cost. The objectives of this study were to evaluate bacterial strains for phenol degradation at different physiological conditions and detection of phenol metabolic pathway. Eight bacterial strains were isolated from different environmental samples through enrichment in MSM containing 100 mg L^{-1} phenol, among them *Lysinibacillus* spp. NIGAB-4 showed good growth on phenol plates and was further assessed to check its phenol mineralization efficiency. This isolate was identified through 16S rRNA gene as a member of the genus *Lysinibacillus*. This strain tolerated up to 1200 mg L^{-1} phenol when supplied as a sole source of carbon and energy and has the potential to degrade 800 mg L^{-1} phenol in 96 h. Phenol (200 and 400 mg L^{-1}) degradation at different temperature, pH, and NaCl was checked. It was found that this strain has the potential to degrade phenol in the range 20–45°C, while optimum degradation was achieved at 30°C. *Lysinibacillus* spp. NIGAB-4 has the potential to degrade these phenol concentrations in the pH range 6–9, while complete phenol degradation was observed at pH 7. This strain was able to degrade 200 mg L^{-1} completely at 0 and 2% NaCl concentrations. However, at 4% NaCl a notable amount of phenol degradation was also observed. Phenol degradation pathway was detected by observing the amplification of catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) gene and PCR amplification of C23O gene actively provided the evidence of phenol degradation via meta-pathway. 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: Phenol; C23O gene; 16S rRNA gene; *Lysinibacillus;* pH; Temperature; NaCl

Introduction

Organic pollutants produced because of industrial and agricultural activities have been one of the hazardous comprehensive issues in current years. Maximum pollutants are added into the environment by industrial waste comprises of organic and inorganic contaminants which has adverse effects on living systems. The release of pollutants into our environment whether inadvertently or due to human activities is the foremost cause of water and soil pollution (Holliger *et al*. 1997; Sachan *et al*. 2019). This drain off of wastewater into the environment also results in the spoil of water used for domestic and agricultural consumptions. Among these pollutants, phenol which is the basic structural unit of a wide variety of synthetic organics (Agarry and Solomon 2008) is grouped as priority pollutant by the United States of environmental protection agency (US-EPA 1979). Phenol is used as a solvent, as an antiseptic and as an additive in disinfectants (Pan and Kurumada 2008). Due to high level utilization of phenol in industries, a remarkable amount is added to the environment and possesses toxicity (Su *et al*. 2018) against all form of life including human, plant and microorganisms. In humans, acute exposure to phenol by the oral route leads to damage to blood, liver, kidney and cardiac toxicity, including weak pulse, cardiac depression, and reduced blood pressure (Mohanty and Jena 2017). Phenol is also harmful to the non-living environment. Effects on the non-living environment include damage to structures by acidic air pollutants, effects on the ozone layer and earth's heat balance and reduced visibility.

Phenol take the 11th place under the 129 chemicals with a discharge limit of lower to one mg L^{-1} in the treated effluent in order to human health protection from the possible toxic effects caused by exposure to phenol (Tor *et al*. 2006). Its removal from environment is mandatory to meet the goal of clean environment. Many methods are in practice for phenol removal from the environment such as incineration, chemical oxidation, adsorption, coagulation, photolytic degradation (Al-Dawery *et al*. 2019), photolytic degradation (Feng *et al*. 2020) and bioremediation (Jaber *et al*. 2020). However, attention is given to bioremediation for the reasons of its environment-friendly nature and less cost as compared with physical or chemical remediation techniques.

Many microorganisms (fungi, bacteria, algae, and archaea) are endowed with phenol degrading property. After bacteria, fungi are well evaluated for phenol degradation. Jiang *et al*. (2015) reported phenol degrading efficiency of a fungus belonged to the genus *Candida* which was isolated from activated sludge collected from a pharmaceutical plant which had the potential to degrade up to 800 mg L^{-1} phenol in 72 h. Another study conducted by Santos and Linardi (2004) explored *Graphium* spp. and *Fusarium* spp. for phenol degrading potential which were able to degrade 75% of 10 m*M* phenol in 168 h. Many algal strains are also reported which are equipped with phenol degrading potential. Cheng *et al*. (2017) employed *Tribonema minus* (Algae) for elimination of 700 mg L^{-1} phenol from industrial effluent and Priyadharshini and Bakthavatsalam (2016) reported phenol degradation by *Chlorella pyrenoidosa*. Among the microorganisms, bacteria are well documented for phenol degradation because of simple structure, simple genome, and less expensive in handling in term of culturing and utilization. A lot of members of bacterial genera are characterized for phenol degrading potential. These genera comprise *Rhodococcus, Bacillus, Lysinibacillus, Pseudomonas, Comamonas, Acinetobacter* and *Halomonas* etc. (Ahmad *et al*. 2015).

Few reports are available on phenol biodegradation at different environmental conditions like pH, temperature, and NaCl and biodegradation in such conditions is grossly neglected in Pakistan. There was a need to isolate bacterial strain from our own environment which are efficient in degrading phenol. Keeping in view this gap the aim of this study was to identify and characterize bacterial strains capable of utilizing phenol at different pH, temperature, and NaCl concentrations.

Materials and Methods

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

Isolation and purification

Industrial waste was collected from Industrial area 1–9, Islamabad (33.6607°N, 73.0639°E). Twenty ml of waste was mixed with 80 mL of mineral salt medium (MSM) broth supplemented with 100 mg L^{-1} phenol as a single energy source, and placed on a shaker at 28°C. MSM was prepared according to Ahmad *et al*. (2015). After seven days of enrichment, 0.5 mL of this sample was spread on MSM agar plates containing 100 mg L^{-1} phenol as a sole source of carbon and energy and incubated at 28°C till visible bacterial growth. After full growth of the colonies, single and pure colonies were picked and streaked on Tryptic Soy Agar (TSA) plates and incubated at 28°C. This process was repeated until the isolation of pure culture. Growth of many strains were observed and named as NIGAB-1, NIGAB-2, NIGAB-3, NIGAB-4 and so on. All the strains were preserved at -80°C in 70% glycerol. Isolated strains were morphologically characterized by observing colony shape, color, opacity, elevation etc. Our strain of interest which showed best growth on TSA plate was NIGAB-4.

Identification and phylogenetic analysis

NIGAB-4 was identified through 16S rRNA gene. On TSA plate NIGAB-4 was first grown. In PCR tubes 20 μL of Tris EDTA (TE) buffer was added, 2–3 few colonies of NIGAB-4 were added, and homogenized using vortex machine. Then the PCR tubes were placed at 95°C for 10 min in a thermal cycler. The culture was centrifuged at 12000 rpm for 10 min and the supernatant was collected and used as template DNA. The 16S rRNA gene was amplified with forward primer 9F (5-GAGTTTGATCCTGGCTCAG-3) and reverse primer 1510R (5-GGCTACCTTGTTACGA-3) followed a PCR program used by Ahmad *et al*. (2015). Amplified products were confirmed by gel electrophoresis (1%) and purified using commercially available DNA purification kit (Invitrogen). Purified products were sequenced using internal primers 27F (5-AGAGTTTGATCMTGGCTCAG-3) and 1492R (5-ACCTTGTTACGACTT-3) from Macrogen, Korea.

The sequences obtained from Macrogen Korea were trimmed in BioEdit software (v. 7.0.9.0.) to get the refined sequences and the contig assembly was constructed in DNA Dragon (v. 1.7.0). The assembled consensus sequence was BLAST (Basic Local Alignment Search Tool) search using ezbiocloud Server (https://www.ezbiocloud.net/) to retrieve sequences of 16S rRNA gene of closely related validly published species for exact identification of our strain. On the basis of maximum identity score, sequences were selected and alignment was performed in Clustal W (version 1.6) (Thompson *et al*. 1994). A phylogenetic tree was created with the use of the Neighbor-Joining algorithm in MEGA-5 (Tamura *et al*. 2011).

Biochemical characterization

Consumption of different carbon sources by the isolated strain was determined using API 20E kit (bioMerieux, France) according to the manufacturer's protocol. NIGAB-4 was first grown on TSA plates. 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 an incubator at 28°C for 24–48 h. The results were recorded according to color change.

Phenol tolerance

NIGAB-4 was tested for its tolerance to various initial concentrations of phenol (0, 200, 400, 600, 800, 1000, 1200, and 1400 mg L^{-1}). This experiment was performed in MSM broth containing phenol as the only carbon source. Blank (MSM broth with phenol without inoculation) of each treatment was run in parallel to determine the bacterial growth. NIGAB-4 was inoculated in ten mL of MSM broth containing phenol and incubated at 30°C. After visible growth, 500 μL aliquot was taken and added to 250 mL flasks containing the aforementioned concentrations of phenol. Growth was checked at the specific interval by taking OD at 600 nm using a spectrophotometer. The experiment was performed in triplicate.

Phenol degradation

Phenol degrading efficiency of the isolated strain was determined in MSM broth augmented with 0, 200, 400, 600, 800, 1000, 1200, and 1400 mg L^{-1} phenol at 30°C and pH 7. Initial reading was taken at 0 h of inoculation and the subsequent readings were taken with the internal of 12 h. One mL of the aliquot was harvested at each interval, centrifuged at 10000 rpm for 10 min. The supernatant was checked for remaining phenol. Two types of control were used in parallel, 1) MSM broth containing phenol (0–1400 mg L^{-1}) and was without inoculum (Abiotic control). Another control was MSM broth without phenol and with bacterial culture (Biotic control). This experiment was performed in triplicate. Phenol was identified on the basis of external standards (50, 100, 150, 300, 500 mg L^{-1}) and residues were quantified using High-Performance Liquid Chromatography (HPLC) according to Ahmad *et al.* (2014).

Optimization of physiological parameter for phenol degradation

At different pH, temperature, and NaCl concentrations, phenol degrading potential of this strain was checked. Two phenol concentrations (200 and 400 mg L^{-1}) were used to determine the optimum temperature, and pH, and 200 mg L^{-1} phenol was used to determine the range of NaCl concentrations in which this strain can degrade phenol.

Temperature optimization

NIGAB-4 was checked for 200 and 400 mg L^{-1} phenol degradation at 0, 15, 20, 30, 37, 40, 45 and 50°C. This strain was first grown in MSM broth (10 mL) containing 100 mg L^{-1} phenol and 500 μL of the grown culture was added in 50 mL of MSM broth at the aforementioned temperatures and at pH 7. Growth was checked by taking OD at 600 nm at a time interval of 12 h by comparing with the OD of respective blanks. At 12 h time interval, one mL of the culture was harvested, centrifuged at 10000 × *g* for 10 min and remaining phenol quantity was determined using HPLC. Abiotic control was run in parallel with each concentration. This experiment was performed in triplicate.

pH optimization

The same experimental design was used for pH optimization, however, in this experiment pH 4, 5, 6, 7, 8, 9, and 11 were used. The optimum temperature for phenol degradation was used in pH optimization. pH range in which this strain can degrade phenol and optimum pH was determined. This experiment was performed in triplicate.

NaCl optimization

Phenol degradation at 0, 2, 4, 6, 8 and 10% NaCl concentration was checked. For this purpose, the above mentioned NaCl concentrations were prepared. Pre-culture of 0.5 mL was inoculated in fifty mL of MSM broth augmented with 200 mg L^{-1} phenol. Optimum temperature and pH determined were used for this experiment. Range and optimum NaCl concentration were determined. Growth was checked by taking OD at 600 nm at a time interval of 12 h by comparing with the OD of respective blanks. At 12 h time interval, one mL of the culture was harvested, centrifuged at 10000 rpm for 10 min and remaining phenol quantity was determined using HPLC. This experiment was performed in triplicate.

Molecular detection of phenol degrading pathway

Genes, C12O and C23O responsible for phenol degradation were determined to check the pathway acquired by the isolated strain. For this purpose, genomic DNA was extracted by CTAB method (William *et al*. 2012). C12O gene was amplified with primers, C12OF (5-GCCAACGTCGACGTCTGGCAGCA-3) and C12OR (5-CGCCTTCAAAGTTGATCTGCGTGGTTGGT-3) and C23O gene was amplified using primers, C23OF (5-AAGAGGCATGGGGGCGCACCGGTTCGA-3), and C23OR (5-TCACCAGCAAACACCTCGTTGCGGTTGCC-3) according to the PCR protocol proposed by Sei *et al*. (1999). Amplified products were visualized on 1% agarose gel. Marker (100 bp) was used for quantification of the bands.

Results

A total of eight bacterial strains were isolated from industrial waste of I-9, Islamabad among which NIGAB-4 was selected for its phenol degrading potential. This strain was gram-positive. Colony morphology was determined and it was found that the shape of the colony was circular with entire margins and rough surface. Colony elevation was crateform with pink color and opaque in nature.

Identification of isolated strains

NIGAB-4 was identified as a member of the genus *Lysinibacillus* the closest phylogenetic neighbors were *Lysinibacillus fusiformis* (AB271743), *Lysinibacillus mangiferihumi* (JF731238) and *Lysinibacillus sphaericus* (AUOZ01000024) which shared 99.49, 98.73 and 98.8 sequence percent identity, respectively (Table 1). Phylogenetic analysis confirmed the affiliation of NIGAB-4 with *Lysinibacillus fusiformis* NBRC 15717T (AB271743) with the Bootstrap value of 98% (Fig. 1). The sequence obtained from Macrogen Korea was submitted to DDBJ with the accession number MH602434. The length of assembly made for NIGAB-4 was 1348 nucleotides.

Biochemical characterization

Table 2 shows the biochemical characterization of NIGAB-4. After 48 h of incubation, the strain NIGAB-4 was weakly positive for *β*-galactosidase and Citrate and positive for Gelatinase, Indole production, and oxidation/fermentation of Glucose Melibiose Saccharose and Sorbitol while negative for utilization of Arginine, production of H_2S, Lysine decarboxylase, Ornithine decarboxylase, Sodium pyruvate, Tryptophan deaminase, Urease, fermentation/oxidation of Amygdalin Arabinose Mannitol and Rhamnose.

Phenol tolerance

NIGAB-4 was incubated for 96 h to check its tolerance to the mentioned concentrations. No growth was perceived at 0, and 1400 mg L^{-1} phenol concentration. A good growth was observed in MSM broth containing 200–1000 mg L^{-1} phenol. Optimum growth was observed at 800 mg L^{-1} phenol. Little growth was noted at 1200 mg L^{-1} phenol. Up to 600 mg L^{-1} phenol, no lag was observed. Maximum growth at 200 mg L^{-1} phenol was recorded after 36 h of incubation (OD = 0.7). After 36 h, decline phase was started and the optical density reduces to 0.27 after 96 h. At 400 mg L^{-1} phenol highest OD was recorded as 0.61 after 60 h. A decline phase was started afterward and the OD value reduces to 0.43 after 60 h of total incubation time. At 600 mg L^{-1} maximum growth (OD = 0.96) was noted at 72 h of incubation. Decline phase initiated after 72 h delineated by the reduction in OD which was 0.78 after 96 h of incubation. At 800 mg L^{-1} phenol, maximum growth was achieved as indicated by OD which was 1.32 after 72 h of incubation and afterward reduces to 0.91 after 96 h. At 1000 mg L^{-1} phenol, a lag phase for 24 h was noted and overall the growth was slow and highest OD (0.66) was observed after 84 h. A lengthy lag phase was observed for 60 h was observed at 1200 mg L^{-1} phenol and the maximum growth achieved having the OD 0.38 after 96 h of incubation. No growth was observed at 1400 mg L^{-1} phenol (data not displayed) (Fig. 2).

Phenol degradation

For quantification of degraded phenol, six external standards were run (50, 100, 150, 300, 500 and 800 mg L^{-1}). Residual phenol in the aliquot was measured by employing the equation achieved from the regression analysis of the external standards, for which the R^2 and adjusted R^2 value were recorded as 98.05 and 96%, respectively (Fig. 3).

No phenol degrading activity was noted at 0, 1400 mg L^{-1} and abiotic control. At 200 mg L^{-1} phenol, NIGAB-4 was incubated for a total period of 60 h and this strain had the capability to completely degrade such concentration in 48 h. Highest degradation was observed at 12 h of incubation at which 46% phenol was mineralized. The R^2 value was recorded as 99.2% and average degradation rate was noted as 3.26 mg L^{-1} h^{-1}. Maximum degradation rate was noted at 12 h of incubation for which degradation rate was 7.73 mg L^{-1}/h. This strain was able to degrade 400 mg L^{-1} phenol 48 h. At 12, 24 and 36 h of incubation, 39.08, 67.24 and 90.16% phenol degradation was recorded, respectively. The R^2 value was recorded as 99.94% and average degradation rate was noted as 6.6 mg L^{-1} h^{-1}. Maximum degradation rate was noted at 12 h of incubation for which degradation rate was 13.02 mg L^{-1} h^{-1}. This strain was capable of degrading 600 mg L^{-1} phenol in 72 h. Highest phenol degradation was observed at 36–48 h of incubation which was about 23% of the total phenol supplemented. The R^2 value for this degradation was recorded as 98.8% and the average degradation rate was noted as 7.09 mg L^{-1} h^{-1}. Maximum degradation rate was noted at 24 h of incubation for which degradation rate was 9.35 mg L^{-1} h^{-1}. No lag phase found for 200 and 400 mg L^{-1} phenol. NIGAB-4 had the potential to degrade 800 mg L^{-1} phenol in 96 h, however, a lag phase of 24 h was observed in which 3.31% phenol mineralization was observed. Highest phenol degradation was observed at 48–60 h of incubation which was about 28% of the total phenol supplemented. The R^2 value for this degradation was 94.7% and average degradation rate was noted as 7.35 mg L^{-1} h^{-1}. Maximum degradation rate was noted at 60 h of incubation for which degradation rate was 18.84 mg L^{-1} h^{-1}. The selected strain was found not capable of degrading 1000 mg L^{-1} and in the entire time course and only 43% of the total phenol was degraded. A lag phase of 48 h was observed in which 6.86% phenol mineralization was observed. A very slow degradation rate was observed which indicates the

Table 1: Identification of NIGAB-4 isolated from industrial waste

Strain	Closest Match	Identity (%)	Coverage (%)	Taxonomy
NIGAB-4 (MH602434)	*Lysinibacillus fusiformis* (AB271743)	99.49	100	Bacteria; Firmicutes; Bacilli; Bacillales; Planococcaceae; *Lysinibacillus*;
	Lysinibacillus mangiferihumi (JF731238)	98.73	98.44	
	Lysinibacillus sphaericus (AUOZ01000024)	98.8	100	
	Lysinibacillus xylanilyticus (LFXJ01000007)	97.84	100	
	Lysinibacillus pakistanensis (BBDJ01000063)	97.82	91.52	
	Lysinibacillus contaminans (KC254732)	97.57	97.22	

Table 2: Biochemical Characterization of NIGAB-4 using API 20E kit

S. No.	Biochemical tests	Result	S. No	Biochemical tests	Results
1	β-galactosidase	w	*Fermentation/oxidation of:*		
2	Arginine dihydrolase	-	12	Amygdalin	-
3	Citrate utilization	w	13	Arabinose	-
4	Gelatinase	+	14	Glucose	+
5	H_2S production	-	15	Inositol	-
6	Indole production	+	16	Mannitol	-
7	Lysine decarboxylase	-	17	Melibiose	+
8	Ornithine decarboxylase	-	18	Rhamnose	-
9	Sodium pyruvate	-	19	Saccharose	+
10	Tryptophan deaminase	-	20	Sorbitol	+
11	Urease	-			

w weakly positive, + positive, - negative

toxicity of this concentration to NIGAB-4. The R^2 value for this degradation was recorded as 95.8% and average degradation rate was noted as 2.76 mg L^{-1} h^{-1}. Maximum degradation rate was noted at 60 h of incubation for which degradation rate was 9 mg L^{-1} h^{-1}. Only 10% phenol degradation was detected at 1200 mg L^{-1} phenol (Fig. 4).

Optimization of physiological parameter for phenol degradation

Temperature optimization: NIGAB-4 was checked for 200 and 400 mg L^{-1} phenol degradation at 0, 15, 20, 30, 37, 40, 45 and 50°C. The bacterial growth was measured by taking optical density at 600 nm. MSM broth containing these two concentrations was inoculated at pH 7. No phenol degradation was recorded at 0, 15 and 50°C. NIGAB-4 was incubated for a total period of 36 h at 200 mg L^{-1} at given temperatures. It was found that this strain has the potential to degrade phenol in the range of 20–45°C. This strain was able to degrade 100% phenol at 30°C where the OD reached to 0.61. At 37°C, 64.5% (OD=0.49) of the total phenol was degraded by this strain followed by degradation at 40°C (58.35%) and at this concentration, the OD was recorded as 0.34. Only 13.61% phenol degradation was observed at 20°C (OD=0.24). At 45°C 11.45% phenol degradation was observed. At 400 mg L^{-1}, this strain was incubated for 36 h and 100% phenol degradation was observed at 30°C

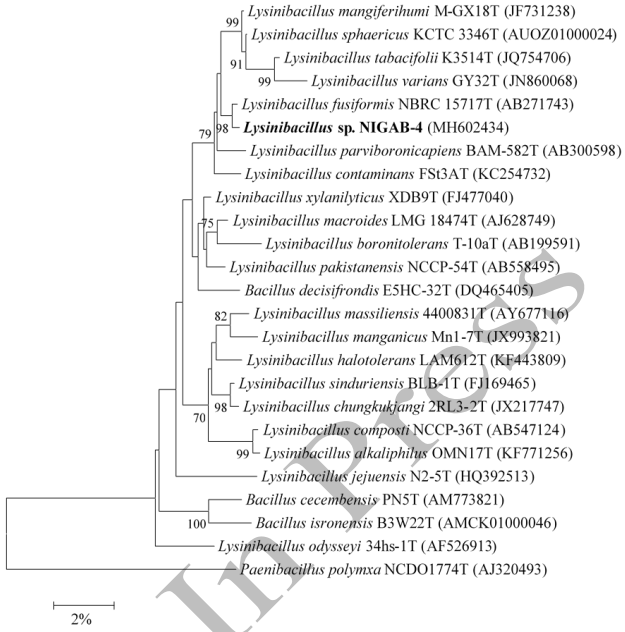

Fig. 1: Phylogenetic tree showing inter-relationship of *Lysinibacillus* spp. NIGAB-4 with closely related species of the genus *Lysinibacillus* inferred from 16S rRNA gene sequences. The tree was generated using Neighbor-joining algorithm contained in Mega 5.0 software package. Bootstrap values (only >70% are shown) expressed as a percentage of 1000 replications. Bar sequence divergence was 2%. *Paenibacillus polymxa* NCDO1774T (AJ320493) was used as a root. Accession number of each type strain is shown in parentheses

(OD=0.74) followed by degradation at 37°C where 73.24% of phenol degradation was observed (OD=0.62). At 40°C 49.53% phenol degradation was recorded where OD reached to 0.57. At 20 and 45°C, 12.059 and 13.02% phenol degradation was recorded, respectively (Fig. 5a).

pH optimization

For pH optimization, NIGAB-4 was subjected to degrade 200 and 400 mg L^{-1} phenol at 30°C and pH 4–10 in MSM broth for a total period of 36 h. NIGAB-4 has the potential to degrade these phenol concentrations in the range of pH 6–9. This strain was able to degrade almost 100% of both phenol concentrations at pH 7. OD reached at 200 and 400 mg L^{-1} phenol was recorded as 0.58 and 0.623 at this pH. At pH 6, NIGAB-4 was able to degrade 534.87% (OD=0.36) and 22.04% (OD=0.46) of 200 and 400 mg L^{-1} phenol, respectively. OD value of 0.401 and 0.48 was achieved at pH 8 where 45.27 and 34.24% of 200 and 400 mg L^{-1} phenol degradation was observed, respectively. At pH 5 negligible amount of phenol degradation was observed while at pH 9 very low phenol degradation was observed. At pH 9, 8.6% (OD=0.18) and 7.4% (OD=0.26) of 200 and 400 mg L^{-1} phenol degradation was observed, respectively (Fig. 5b).

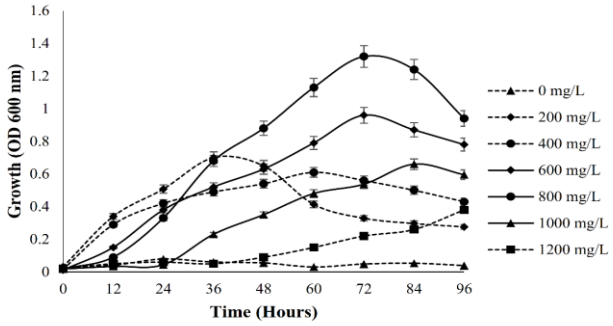

Fig. 2: The growth of NIGAB-4 in MSM supplemented with different phenol concentrations (0-200 mg L^{-1}). Blank of each concentration was used for comparing growth

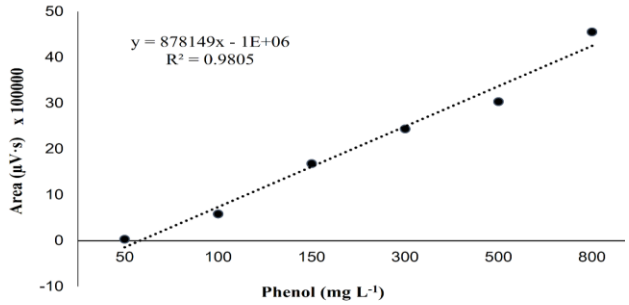

Fig. 3: External standers of phenol used for quantification of degraded phenol

NaCl optimization

NIGAB-4 was incubated at pH 7 and 30°C in 200 mg L^{-1} phenol with the aforementioned concentration of NaCl for 84 h. The strain was able to completely degrade 200 mg L^{-1} phenol at 0 and 2% NaCl. At 0% NaCl, 200 mg L^{-1} phenol was degraded in 48 h, where the maximum value of OD was reached 0.53. No lag phase was found at this NaCl concentration. At 2% NaCl, this strain was able to degrade 200 mg L^{-1} in 72 h. The lag phase of 12 h was recorded. The highest OD was recorded at 48 h of incubation and a stationary phase was observed between 48–60 h of incubation. At 4% NaCl, 69.5% of 200 mg L^{-1} phenol degradation was observed in the entire time course. The lag phase of 24 h was noted. The highest growth was recorded at 60 h of incubation where the OD reached to 0.27. After 72 h decline phase was initiated. At 6% NaCl, only 45 mg L^{-1} phenol of 200 mg L^{-1} degradation was recorded. A lag phase of 36 h was observed. Highest OD was obtained at 48 h of incubation where OD reached to 0.14. Afterward decline phase was started. At the rest of NaCl concentrations, no phenol degradation was observed (Fig. 6).

Molecular detection of phenol metabolic pathway

The aim of this experiment was to determine the pathway through which phenol is catabolized by the

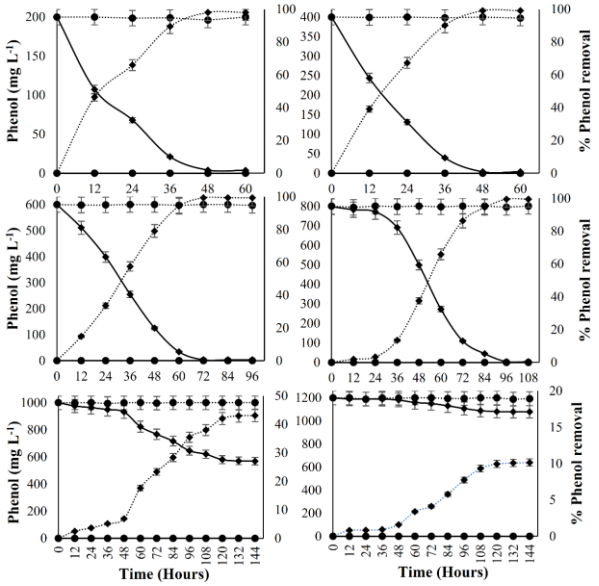

Fig. 4: Degradation of phenol (200 - 1200 mg L^{-1} phenol) in MSM broth and percent degradation of NIGAB-4 with respect to time. Phenol (0 mg L^{-1} Abiotic control Phenol degradation Percent Removal)

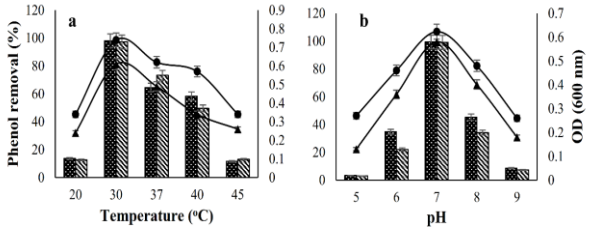

Fig. 5: Percent removal of 200 and 400 mg L^{-1} phenol by NIGAB-4 at different temperatures (**a**) and pH (**b**). Phenol 200 mg L^{-1} Phenol 400 mg L^{-1} Growth at 200 mg L^{-1} phenol Growth at 400 mg L^{-1} phenol . Growth is given in term of optical density.

presence/absence of C12O and C23O genes. No amplification was observed of C12O and C23O genes. After running the amplified products on a gel, the band for C23O gene was observed which was 900 bp. This size is the characteristic size of the amplified product of this gene using the primers mentioned in materials and methods section. The absence of band in control (negative control) showed the authenticity of this experiment (no contamination). This observation showed that all the strains acquired meta-pathway for phenol degradation.

Discussion

Phenol degrading bacteria are naturally found in diverse habitat including soil (Gayathri and Vasudevan 2010), plant leaves (Sandhu *et al*. 2009), activated slug, waste-water

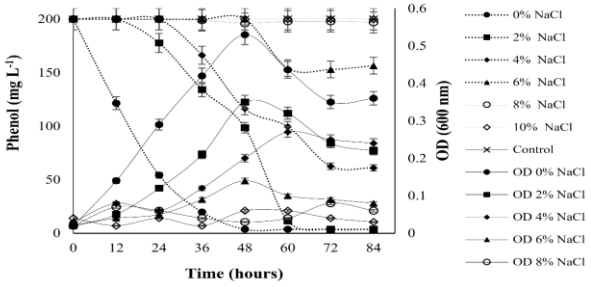

Fig. 6: Degradation of 200 mg L^{-1} phenol by NIGAB-4 at different concentrations of NaCl

(Banerjee and Ghoshal 2010), industrial waste (Ahmad *et al*. 2015) and water (Tambekar *et al*. 2013). The presence of phenol-degrading bacteria in such diverse habitats provides the evidence of the widespread distribution of this trait. Among the eight morphologically and biochemically distinct bacterial strains, *Lysinibacillus* spp. NIGAB-4 was selected for phenol degradation because of its good growth after enrichment in phenol containing MSM plates. Source and physiological parameters of samples affect the efficiency of bacterial strains regarding phenol degradation. Efficient phenol degraders can be isolate from the sources where phenol or its derivatives are present. For isolation of our strains, we selected industrial waste which is reported to have the maximum chances of the existing of hydrocarbon-degrading bacteria and the persistent bacterial strains are normally well-adopted (Hamitouche *et al*. 2012). Majority of bacterial strains present in the environment are endowed with phenol degrading property while the ability of some strains is enhanced through enrichment in phenol containing medium. This technique enhances the reduction of phenol toxicity through adaptation and the potential of biodegradation. This eminence is naturally gifted because of the presence of genes in bacterial genomes allow bacteria to withstand with stress conditions (Alexander 1999).

The strain used in this study was identified on the basis of 16S rRNA gene. This technique is the benchmark for identification bacterial species nowadays. Gürtler and Mayall (2001) stated that sequence analysis of the genes encoding rRNA, usually 16S rRNA, has been well-established as a standard procedure for the identification of bacteria at the levels of species, genera, and family. The exact taxonomic position of bacteria is extensively determined on the basis of 16S rRNA gene to avoid ambiguity produced through identification only on morphological characteristics. This method is preferred over other methods because of some advantages including the presence of this gene in all bacteria length of this gene is enough to get information, conservation of gene function and the availability of a huge computerized database for comparing the query sequence (Patel 2001). The databases used in this study are DDBJ and Ez-Taxon server. The Ez-Taxon server was preferred because of containing the sequences of validly published bacterial species.

NIGAB-4 was able to tolerate 1000 mg L^{-1} phenol. To some extent this strain possess tolerance up to 1200 mg L^{-1} phenol, however, the growth was inhibited when 1400 mg L^{-1} phenol was used. Bacterial resistance to high phenol concentration is considered as natural or change and mutation in the gene pool (Ajaz *et al.* 2004). May be horizontal gene transfer plays a role in this regard. Bacteria possess tolerance to pollutant to ensure its survival in unfavorable conditions. Tolerance to phenol by bacteria is well documented and a lot of bacterial strains are reported which are able to tolerate phenol (Pradeep *et al*. 2015). At high concentration of phenol, bacterial growth is inhibited due to substrate toxicity. This may be due to low production of bacterial own growth metabolites at high concentration of phenol.

Phenol degrading ability of NIGAB-4 was checked at different concentrations of phenol. This strain had the potential to degrade completely maximum 800 mg L^{-1} phenol in 96 h. Only 10% of 1200 mg L^{-1} phenol was degraded and no degradation occurred when 1400 mg L^{-1} phenol was used as the only energy and carbon source. With the increase in phenol concentration, degradation efficiency of this degraded due to substrate toxicity. Our results are substantiate with the that of Jame *et al*. (2010) with a little variation who determined the degradation of 800 mg L^{-1} phenol in 72 h by a strain *Pseudomonas* FA. Degradation time of a particular phenol concentration by the member of different genera or even species of the same genus may be different which is not surprising. For Instant, *Pseudomonas* FA degraded 800 mg L^{-1} phenol in 72 h while, other members of this genus (*Pseudomonas* SA, TK and KA) was unable to degrade such amount of phenol even in 96 h Jame *et al*. (2010). strains of the *Lysinibacillus* genus are reported for phenol degradation (Lin *et al*. 2010; Ahmad *et al*. 2015). *Bacillus fusiformis* (now *Lysinibacillus fusiformis* Ahmed *et al*. 2007) is also reported for phenol degradation in the presence of iron-based nano-particles (Kuang *et al*. 2013). Similarly, phenol tolerance and phenol degradation may not be confused. A strain will tolerate a particular amount of phenol but it is not necessary to degrade that amount. For example, Liu *et al.* (2016) reported the degradation of 800 mg L^{-1} phenol by *Acinetobacter calcoaceticus* but can grow up to 1700 mg L^{-1}.

Physiology parameter like Temperature, pH, and NaCl concentration was optimized for phenol degradation. It was found that temperature 37°C, pH 7 and 0% NaCl were optimum for this strain to degrade phenol.

The temperature was optimized for phenol degradation for this strain when 200 and 400 mg L^{-1} phenol was employed. Temperature 30°C was found optimum for the degradation of such concentrations. The second highest degradation rate was recorded at 37°C followed by 40°C. Temperature optimization for phenol degradation is not yet reported for this strain however, the phylogenetic neighbors of our strain are reported for degradation of other organic compounds for which the optimum temperature was

recorded as 30°C. Chantarasiri and Boontanom (2017) reported lignin degradation by *Lysinibacillus sphaericus* for which the optimum temperature was 30°C. For naphthalene degradation by *Bacillus fusiformis* (BFN), optimum temperature was recorded as 30°C. At lower and higher temperatures than 30°C, reduction in biodegradation of phenol was observed. At higher temperature, metabolic activities are reduced due to less solubility of O_2 (Bamforth and Singleton 2005). Reduction in phenol degradation below and above 30°C designates that NIGAB-4 is mesophilic in nature. Bayoumi and Abdul-Hamd (2010) reported 30°C as the optimum temperature for phenol degradation by a bacterium.

NIGAB-4 was able to degrade 200–400 mg L^{-1} phenol in pH rang of 6–9 in which the optimum pH was 7. Phenol degradation at different pH values are not yet reported for this strain, however, normal growth on TSA medium under different pH was determined and the optimum was found as 7 (Priest *et al.* 1988). So, finding microbes for their best degradation performance in this range is not astounding (Mentzer and Ebere 1996). At pH 8, 45 and 35% of 200 and 400 mg L^{-1} was degradation respectively, which shows the tendency of this strain towards the alkaline environment. Phenol degrading efficiency decreased below and above the neutral pH. This is due to the dependency of phenol degradation on pH which affects the surface charge of the absorbent and the degree of ionization (Annadurai *et al.* 2000). It is observed that neutral pH favor bacterial growth. The reason behind the phenomenon is the stability and efficiency of most enzymes at neutral pH. Change in pH results in a minimum or complete loss of enzymatic activity. Another theory revealed that at extreme low or high pH acids or bases enter the bacterial cells easily because they tend to exist in un-dissociated form under these conditions and electrostatic force cannot stop them from inflowing to bacterial cells, which in turn affects the metabolic pathway of the organism (Robertson and Alexander 1992; Karigar *et al*. 2006).

NIGAB-4 was able to completely degrade 200 mg L^{-1} phenol at 0 and 2%. Degradation at 0% salinity shows the activeness of this strain. With the increase in NaCl concentration from 2–4% the growth of this strain as well as phenol degradation reduced. At 6% NaCl, only 45 mg L^{-1} phenol was degraded and no phenol degradation was observed on onwards concentration. This study showed the efficient degradation of 200 mg L^{-1} phenol, however, at high salinity level, no phenol degradation was observed which proved the toxicity of NaCl. This may be due to the restriction in metabolism (Jiang *et al.* 2015). Phenol degradation by *Lysinibacillus* spp. under different NaCl concentrations is not yet reported, however, tolerance to NaCl in nutrient broth was checked by Tomova *et al*. (2014) who reported the tolerance of a *Lysinibacillus* spp. up to 10% NaCl and was considered as moderately halotolerant.

Phenol degradation results in the final products by two pathways *i.e*., Ortho and Meta pathway (Hill and Robinson 1975; Paller *et al.* 1995). This pathway can be determined either by detection of specific intermediates or by detection of genes involved in phenol degradation. In this study, we observed the presence/absence of two genes named C12O and C23O. No amplification of C12O gene was observed while, C23O gene was amplified. A band size of nearly 900 bp was obtained which is the characteristic size for C23O (Hesham *et al*. 2014). The C23O gene amplification was observed which indicates that this strain degrades phenol via meta-pathway.

Conclusion

In this study, we report the isolation, enrichment, molecular identification, and phenol degrading potential of a *Lysinibacillus* spp. Moreover, phenol degradation at various pH, temperature and NaCl concentrations was also carried out. NIGAB-4 the closest phylogenetic neighbor of which is *Lysinibacillus fusiformis* (AB271743) is not yet reported for phenol biodegradation. This strain tolerated 1200 mg L^{-1} phenol and has the potential to degrade 800 mg L^{-1} phenol as a sole source of carbon and energy. Optimum pH and temperature for 200–400 mg L^{-1} phenol degradation were 7 and 30°C, respectively. This strain was able to degrade 200 mg L^{-1} phenol at 2% NaCl and the optimum concentration was 0%. This strain adopted meta-pathway for phenol degradation being carried C23O gene. This strain is helpful in bioremediation of phenol where there is a fluctuation of physiological parameters.

Author Contributions

MRK and NA conceived idea and designed the study. NA performed all the experiments. NA, MRK and GMA analyzed the data. NA and MRK compiled the data and wrote the manuscript. MRK provided the overall supervision and GMA provided the space.

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