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Molecular Phylogeny and Biodegradation of Phenol by Bacteria Isolated from Different Wastewater Environments of Sri Lanka

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ABSTRACT

Biodegradation of phenol has been demonstrated by different bacterial species around the world. The present study focused on the isolation aerobic phenol degrading bacteria from different wastewater environments of Sri Lanka. Bacteria were isolated from wastewater samples in Mineral Salt Media enriched with phenol as the sole carbon source. Identification of bacteria and development of molecular phylogenetic relationships were done based on the 16S rRNA gene sequences. Phenol degradation of identified bacteria was assessed by 4-aminoantiphyrine direct spectrophotometric method. Fourteen different bacterial species; *Klebsiella pneumoniae* (KY967368), *Klebsiella variicola* (MK182938) *Enterobacter asburiae* (MH027514), *Staphylococcus pasteuri* (MG815139), *Staphylococcus warneri* (MG818755), *Ochrobactrum intermedium* (MH645753), *Ochrobactrum oryzae* (MH031688), *Ochrobactrum grignonense* (MK182941), *Alcaligenes aquatilis* (MH636879), *Alcaligenes faecalis* (MH031755), *Pseudomonas aeruginosa* (MH031762), *Pseudomonas monteilii* (MH636875), *Pseudomonas* sp. (MH027519) and *Fictibacillus rigui* (MH036000) were identified. *P. aeruginosa* and *F. rigui* degraded 1800 mgL^{-1} phenol within 120 h and 168 h respectively. Bacterial isolates of *P. monteilii, Pseudomonas* sp., *A. faecalis, A. aquatilis*, *O. intermedium*, *O. grignonense*, *O. oryzae* and, *K. variicola* degraded 1700 mgL^{-1} phenol in ≤ 144 h. The rest of the bacterial species degraded only 1600 mgL^{-1} phenol in ≤ 144 h. All the identified phenol degrading bacterial isolates were from the phyla of Proteobacteria and Firmicutes. Our findings demonstrate the potential of using phenol degrading bacteria in future bioremediation protocols.

1. Introduction

Phenol is a naturally occurring compound that is also released as a pollutant along with solid waste and wastewater. Phenol is mainly discharged to the environment through industrial effluents of pulp mills, coal mines, gasoline, other petrochemical plants, detergents, disinfectants, pharmaceuticals, textiles and dye industries [1-3]. Phenol has been listed as a priority pollutant by the U.S. Environmental Protection Agency (EPA) and the National Pollutant Release Inventory (NPRI) of Canada [4]. Phenol has been reported as genotoxic, immunotoxic and, physiological effects and carcinogenic [5]. Owing to these adverse effects of phenol, the World Health Organization has set a guideline of 1 μgL^{-1} in drinking water. Meanwhile, EPA has established a maximum phenol concentration as 0.5 mgL^{-1} in wastewater disposed into natural water bodies or municipal sewerage systems [6]. Hence, treating wastewater for phenol has become an internationally recognized industrial standard.

A number of physico-chemical methods are currently applied to remove phenol from wastewater. However, bioremediation has been proved as the most appropriate method due to its

efficiency and environmentally friendly nature. Bioremediation is using of microorganisms and their catabolic enzymes for degrading complex contaminants into simple and nontoxic forms.

Despite the bactericidal effect of phenol, many microorganisms are known to degrade phenol to meet their carbon and energy need [7, 8]. Bacterial species such as *Pseudomonas putida* [9-11], *Pseudomonas aeruginosa* [12-14], *Pseudomonas fluorescens* [14], *Alcaligenes faecalis* [15], *Acinetobacter* spp. [16, 17], *Klebsiella* spp. [18, 19], *Corynebacterium* spp. [20] and many others are known for their phenol degradation ability. However, our understanding of the efficacy of phenol degrading bacteria living under higher phenol concentrations with high metabolizing activity as bioremediating agents is poor [21]. Thus, isolation of bacteria growing under higher levels of phenol holds promise for identifying bioremediating agents capable of operating in high phenol concentrations. In addition, identification of locally inhabiting bacterial isolates which are effective at degrading phenol under prevalent environmental conditions is significant for the success of any bioremediation strategy. Therefore, the present study is focuses on isolating and identifying bacteria from different wastewater environments of Sri Lanka and exploring their efficacy in degrading at high concentrations of phenol.

2. Material and Methods

2.1. Collection of wastewater samples

Wastewater samples were collected from different locations in four districts (Colombo, Gampaha, Kurunegala, and Kandy) of Sri Lanka (Table 1). Sampling locations were selected based on the sources of contaminations which were decided by physically visiting the sampling site (Table 1). All the wastewater samples were collected from the identified locations as per the guidelines of EPA [22]. The collected samples were transported in ice to the laboratory and stored at 4 ^{0}C until inoculation.

2.2. Isolation of potential phenol degrading bacteria

Isolation of bacteria was done in the enriched mineral salts medium (MSM) containing (gL^{-1}): K_2HPO_4 2.75 g, KH_2PO_4 2.25 g, $(NH_4)_2SO_4$ 1 g, $MgCl_2.6H_2O$ 0.2 g, NaCl 0.1 g, $FeCl_3.6H_2O$ 0.02 g, $CaCl_2$ 0.01 g at pH 7 [23] and phenol as the sole carbon source. Initially, 10 mL of wastewater sample was inoculated to 90 mL of liquid growth media containing 200 mgL^{-1} phenol and incubated for 7 days at 130 rpm and room temperature [23].

Subsequently, each bacterial culture (10 mL) was transferred to 90 mL of fresh MSM with the same phenol concentration and incubated overnight under the same incubation conditions. The bacteria grown in overnight culture were isolated further on spread plates. Pure cultures of bacteria were isolated by repeated subculturing in phenol (200 mgL^{-1}) MSM solid media.

2.3. Identification and molecular phylogeny of bacteria by 16S rRNA sequence analysis

Genomic DNA extraction was done according to the modified DNA extraction protocol developed by Chauduri et al., (2008) [24]. The full length of 16S rRNA gene were amplified using universal primers, 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (GGYTACCTTGTTACGACTT). PCR reaction mixture was prepared by adding approximately 50 ng of template DNA, 1 x Green Go Taq Flexi buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of forward and reverse primers and 1 U of Green Go Taq DNA polymerase. Thermal cycling parameters were set to 94 ^{o}C for 5 min; 30 cycles of denaturation at 94 ^{o}C for 1 min, annealing at 54 ^{o}C for 1 min, extension at 72 ^{o}C for 2 min and final extension at 72 ^{o}C for 10 min. Amplified products of Polymerase Chain Reaction (1490 bp) were sent to Macrogen DNA sequencing service, Korea for bidirectional sequencing. Homology search of sequences was performed by using the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI). All the nucleotide sequences were deposited in the NCBI nucleotide sequence database (GenBank) and respective accession numbers were retrieved.

Further, molecular phylogenetic relationships of identified bacteria based on 16S rRNA gene sequences were analysed based using Molecular Evolutionary Genetics Analysis software, version 7 (MEGA 7). The unrooted tree was developed by the neighbor-joining method. The data set was bootstrapped 1000 times to increase the reliability of the cladogram.

2.4. Phenol degradation by identified bacteria

Pure cultures of bacteria were initially transferred to liquid MSM with 500 mgL^{-1} phenol and subsequently, to higher phenol concentrations (1000, 1200, 1300, 1400, 1500, 1600, 1700, 1800 and 1900 mgL^{-1}) until there is no detectable growth of bacteria. The phenol concentration penultimate to the concentration which arrests the bacterial growth was recorded as the maximum tolerance level of phenol of the respective bacterial isolates. A stock culture of each isolate was maintained at their maximum phenol tolerance level.

Bacterial inocula for testing the phenol degradation ability of identified bacteria were obtained from the stock cultures at the log phase (OD = 0.7). The bacterial cells were collected by centrifuging at 13,000 rpm for 1 min and washed twice with MSM. Then, the bacterial pellet was resuspended in 1 mL of MSM and transferred to 99 mL of fresh MSM containing phenol. Three replicates of each culture were maintained with a control sample without any bacterial inoculation.

Residual phenol in the medium was measured at 24 h intervals by 4-aminoantiphyrine direct spectrophotometric method as an indirect measurement of phenol degradation following the protocol given in the standard methods for the examination of water and wastewater [25]. Phenol concentration was calculated by using the derived standard equation y = 0.1321x + 0.001 (where y is the optical density at 510 nm and x is the concentration of phenol).

3. Results and Discussion

3.1 Identification and molecular phylogeny of bacteria

The identified bacterial species from different wastewater environments are given in Table 1 with their respective accession numbers in the GenBank. Based on the homology search results of 16S rRNA gene sequences, it was confirmed that the isolated and identified bacteria are from the genera of *Klebsiella, Enterobacter, Staphylococcus, Ochrobactrum, Pseudomonas,* and *Fictibacillus* (Table 1).

Further, the molecular phylogeny of the confirmed bacterial species was found to be from two major phyla of Proteobacteria and Firmicutes (Figure 1). *Klebsiella* spp., *Enterobacter* sp. and *Pseudomonas* spp. were clustered together as they are α Proteobacteria. Meanwhile, two *Alcaligenes* spp. were clustered together in β Proteobacteria subphylum and *Ochrobactrum* spp. in the subphylum of α Proteobacteria.

Table 1 – Isolated and identified bacterial species from different wastewater environments

Location	Nature of the contamination	Isolated and identified Bacteria
Water canal adjacent to Ceylon Petroleum Storage Terminals Ltd. (CPSTRL), Kolonnawa	Oil and petroleum hydrocarbon wastes	*Klebsiella pneumoniae* (KY967368) *Enterobacter asburiae* (MH027514)
Water canal adjacent to the oil refinery, Sapugaskanda	Oil and petroleum hydrocarbon wastes	*Klebsiella variicola* (MK182938) *Staphylococcus pasteuri* (MG815139) *Staphylococcus warneri* (MG818755)
Water canal adjacent to the hospitals, Narahenpita	Hospital wastes	O*chrobactrum intermedium* (MH645753) *Ochrobactrum oryzae* (MH031688) *Pseudomonas* sp. (MH027519)
Wastewater canal adjacent to the Pannala industrial zone, Kurunegala	Industrial wastes of fabrics and dyes	*Fictibacillus rigui* (MH036000)
Wastewater canal adjacent to the bus terminal, Negombo	Urban runoff	*Ochrobactrum grignonense* (MK182941)
Wastewater canal, Kurunegala	Urban runoff and domestic wastes	*Pseudomonas monteilii* (MH636875)
Water drain adjacent to thebus depot, Kuliyapitiya	Oil and urban runoff	*Pseudomonas aeruginosa* (MH031762)
Wastewater canal adjacent to the bus depot, Kandy	Oil and urban runoff	*Alcaligenes aquatilis* (MH636879) *Alcaligenes faecalis* (MH031755)

Isolates identified as *F. rigui* and *Staphylococcus* spp. were grouped together and separated from other species as they are Firmicutes.Therefore, the results indicate that the selected wastewater environments of Sri Lanka harbour diverse potentialphenol biodegrading bacterial species with differentevolutionary relationships.

Further, many research studies reported that most of the phenol biodegrading bacteria were found to belong in the phyla mentioned above [26-28]. The study on community structure and physiological state in the industrial bioremediation system based on the whole-cell hybridisation system proved that predominant bacteria of phenol biodegradation were from Proteobacteria [26].

In addition, phenol degrading bacteria from different Egyptian ecosystems reported by Abd-El-Haleem et al. (2002)have also belonged to the phyla of Proteobacteria and Firmicutes [27]. Further, oil degrading bacteria belonging to the above two phyla have been isolated from coastal waste environments of Sri Lanka [29].

3.2. Phenol degradation by the identified bacteria

In the present study, all the tested bacteria for their phenol degradation were found to degrade 1600, 1700 and, 1800 mgL^{-1} phenol within ≥168 h and considered as low, medium and, high-level degraders respectively. However, the rate of phenol degradation varied among the different bacterial species (Figure 2).

The heterogeneity of the phenol degradation efficiency could be due to the individual genetic makeup of the bacteria which may have the potential to grow in different concentrations of phenol and contain divergent metabolic mechanisms to utilize phenol.

- Bacteria degrading low concentration of phenol

Bacterial isolates of *S. pasteuri, S. warneri* and, *K. pneumonias* howed the fastest degradation of 1600 mgL^{-1} phenol within 120 h (Figure 2a). It has also been reported that *S. pasteuri* was able to degrade other aromatic hydrocarbon like naphthalene [30]. Moreover, *S. warneri* has been identified as an efficient biodegrading agent of phenanthrene, pyrene, and benzo[a] anthracene like polyaromatic hydrocarbons [31]. Kafilzadeh et al. (2010) reported a *Klebsiella* sp. that degraded 700 mgL^{-1} phenol [32]. In addition, *Klebsiella* spp. were reported to exhibit all the characteristics of an efficient phenol degrading microorganism [7]. Further, the present study proved that *E. asburiae* was able to degrade the same concentration of phenol but with a longer duration (144 h) (Figure 2a).

E. asburiae has also been reported as a phenol degrading and biofilm-forming bacteria by an another study [33]. Hence, it further confirmed the phenol degrading ability of *E. asburiae.*

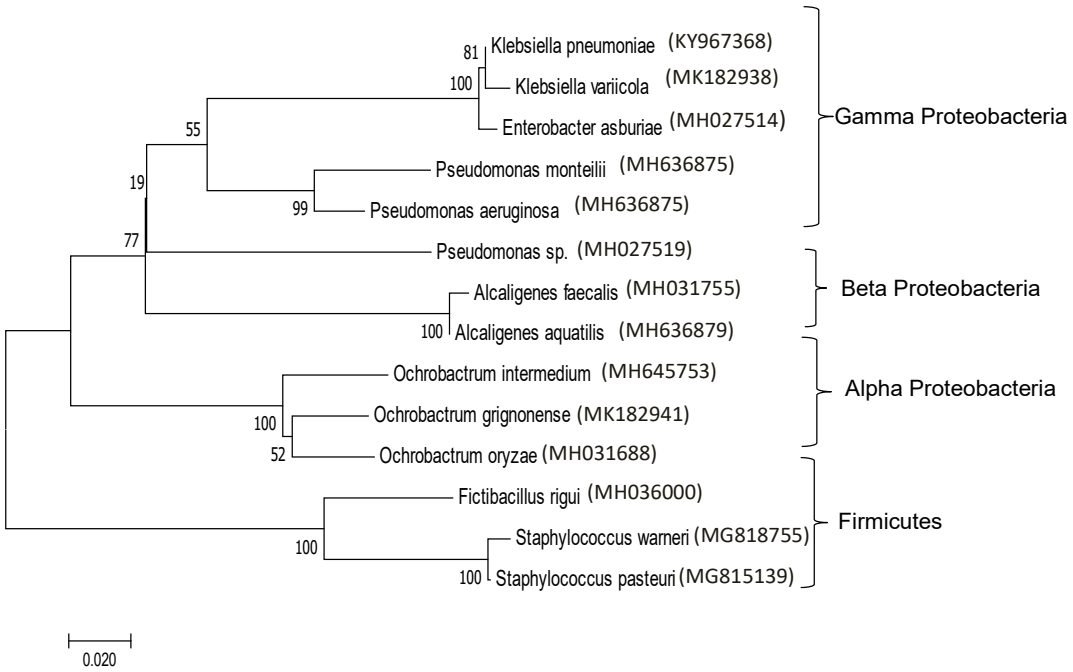

Figure 1: Phylogenetic relationship based on 16S rRNA sequences of isolated bacteria from wastewater

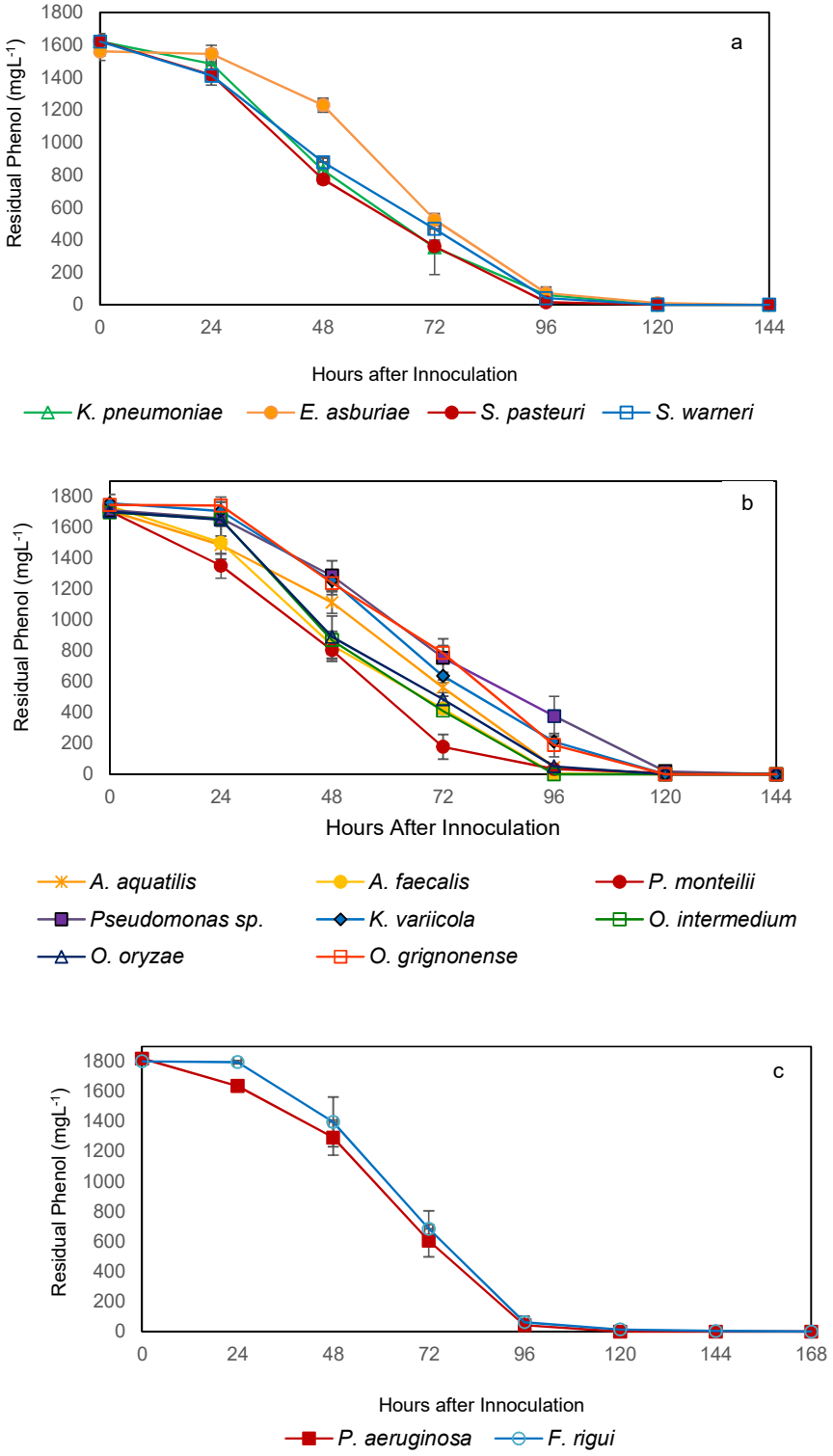

Figure 2- Phenol degradation by identified bacteria in liquid mineral salt media(a) degradation of low concentration of phenol; (b)degradation of medium concentration of phenol;(c)degradation of high concentration of phenol.

The mean value of three replicates and standard deviation by error bars are shown.

- Bacteria degrading medium concentration of phenol

The bacterial isolate of *A. faecalis* resulted in the fastest degradation of 1700 mgL^{-1} phenol within 96 h (Figure 2b) in this study. In supporting the phenol degradation ability of this bacteria, a research study reported a strain of *A. faecalis* which could degrade 1600 mgL^{-1} phenol completely within 76 h [34].Additionally, an isolate of *A. faecalis* has proved to enhance the phenol degradation by immobilizing to spent tea activated carbon [15].

Few more bacterial species; *A. aquatilis, P. monteilii, O. intermedium, O. oryzae, K. varricola* were shown degradation of 1700 mgL^{-1} phenol within 120 h (Figure 2b). Whereas *O. grignonense* and *Pseudomonas* sp. recorded slow degradation of 1700 mgL^{-1} phenol (144 h) compare to other bacteria in this category (Figure 2b). Several other studies have recorded the phenol degradation ability of *Ochrobactrum* sp. [35]. The present study confirmed the phenol degradation ability by three different *Ochrobactrum* spp. indicating the detection within this genus for the same capacity of phenol degradation. Further, a research study reported an efficient phenol biodegradation ability of *Ochrobactrum* sp. CH10 which was isolated from soil in a constructed wetland of China [36].

Additionally, *Ochrobactrum* spp. were also reported to degrade many other petroleum hydrocarbon contaminants, including aromatic hydrocarbons [37, 38], anthracene, phenanthrene, naphthalene, fluorene, and pyrene [39].

- Bacteria degrading high concentration of phenol

Only two bacteria isolated in the present study showed phenol degrading at higher concentration (1800 mgL^{-1}). The local strain of *P. aeruginosa* was found to degrade 1800 mgL^{-1} phenol in 120 h (Figure 2c). Another research study reported *P. aeruginosa* MTCC 4997, isolated from effluents of petrochemical industries to tolerate phenol up to 1400 mgL^{-1} [40]. In parallel, Ghaima et al. (2017) confirmed the degradation of 92% of the 500 mg/L phenol within 48 h and tolerance of 1400 mg/L phenol by another isolate of *P. aeruginosa* [13].

Further, the present study confirmed that *F. rigui* was able to degrade 1800 mgL^{-1} of phenol within 168 h (Figure 2c). However, there are no previous literature records on the biodegradation of phenol by *F. rigui*. Since the present study confirmed the phenol degradation of *F. rigui*, and this could be considered as a new finding of the study.

In summary, the present study was able to identify fourteen different potential phenol degrading bacterial species. Although diverse phenol degrading bacterial community has been reported to degrade phenol, relatively little is known on phenol degrading bacteria in Sri Lanka. Therefore, to the best of knowledge of the authors, this is the first record of identification of bacterial species with the confirmed evidence of their phenol degradation capacities. Moreover, most of the isolated bacteria in the present study were well recorded as the efficient phenol biodegrading agents in the previous literature [13, 31, 32, 34, 35] and those have also been reported to degrade other petroleum hydrocarbons [29, 30, 36, 37, 38].

4. Conclusions

In conclusion, this study confirmed the biodegradation of phenol by fourteen different bacterial species isolated from different petroleum contaminated wastewater environments of Sri Lanka. Tolerance to higher concentrations at different levels and biodegradation of phenol by the identified bacteria proved their potential to be used as phenol biodegrading agents. However, future scale-up studies are needed to employ these bacterial strains in industrial biological treatment systems to treat phenol contaminated wastewater.

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