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# Diversity of bacterial phenol hydroxylase-encoding genes from gasoline-contaminated silt soil

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**Abstract.** *Vasandani SR, Tan WA. 2022. Diversity of bacterial phenol hydroxylase-encoding genes from gasoline-contaminated silt soil. Biodiversitas 23: 5502-5506.* Phenol is an aromatic compound often used as a raw material or intermediate in various industries. Improper handling and disposal may lead to the accumulation of this hazardous compound. Bioremediation is the most viable method to remove phenol from contaminated environment. In this study, the diversity of genes that encode for phenol hydroxylase, a key enzyme in phenol degradation, was assessed in gasoline-contaminated soil from a commercial gas station in Central Jakarta, Indonesia. Partial phenol hydroxylase-encoding gene library was constructed using a pair of universal primer in the pGEM®-Teasy vector. A total of 30 recombinant clones were obtained and sequenced to analyze the genetic diversity of this gene. Obtained clones were 86-99% identical to phenol hydroxylase-related proteins. Phylogenetic tree analysis on amino acid sequences derived from our library revealed that 56.7% of the cloned fragments were closely related to Proteobacteriodota and 20.0% of them were clustered with Actinomycetota. The rest 23.3% of the clones formed a cluster separate from any of the reference sequences, possibly indicating the presence of novel phenol hydroxylase genes.

**Keywords:** Bioremediation, gene diversity, phenol, phenol hydroxylase

## INTRODUCTION

Phenol and its derivatives can be found in the environment as plant secondary metabolites, a product of the anaerobic catabolism of lignin-rich plant materials in ruminant excretion, and as side products in the decomposition of organic matter (Fini et al. 2021). They are also introduced to the environment due to various anthropogenic activities such as the production of dyes, pesticides, insecticides (Anku et al. 2017), and drugs (Zhao et al. 2018), mostly detected in industrial wastewater (Surkatti and El-Naas 2017). These compounds are chemically stable and resistant to degradation, thus, making them persistent in the environment. Phenol itself is highly corrosive and may cause irritation upon contact with skin and respiratory tract. Prolonged exposure to phenol may cause dermal inflammation, necrosis, and affect the nervous system, kidney, lungs, and liver (Anku et al. 2017). Improper handling and disposal results in the accumulation of these compounds in the environment (Duan et al. 2018).

Several physicochemical-based processes have previously been used to help counter and control the ever-increasing levels of phenol in the environment. However, these methods have proven to be time consuming, inefficient, costly, and often lead to the formation of hazardous byproducts (Thakurta et al. 2018). On the other hand, bioremediation using microorganisms that can assimilate to phenol and mediate complete degradation is perceived potential to tackle said limitations and further reduce phenol contamination from the environment.

Bacteria capable of utilizing phenol and its derivatives as their sole carbon, nitrogen and energy sources have been successfully isolated from various environments, such as *Pseudomonas putida*, *Pseudomonas cepacia*, *Bacillus brevis*, *Alcaligenes eutrophus* (Krastanov et al. 2012), *Bacillus badius* D1 (Sarwade and Gawai 2014), and *Rhodococcus opacus* 1CP (Emelyanova and Solyanikova 2020). These bacteria degrade phenol under aerobic conditions using phenol hydroxylase, a ring hydroxylating oxygenase, which adds a hydroxyl group to the benzene ring and forms catechol. Catechol dioxygenase then cleaves the aromatic ring structure of catechol either via the *meta*- or *ortho*- pathway. Through a series of reactions, products formed through both pathways will consequently enter the TCA cycle to produce energy in the form of ATP (Nešvera et al. 2015).

The soil environment is abundant with microbes capable of degrading various anthropogenic substances, including phenol (Fierer 2017). Phenol-degrading bacteria have been recovered from different types of pristine and contaminated soil (Dong et al. 2008; Bui et al. 2012; Wu et al. 2018). However, culture-based approach has its own limitations as only 1% of known microorganisms can be grown in laboratory conditions (Bodor et al. 2020). In the past, phenol hydroxylase gene-based analysis has been used to assess the diversity of bacteria that may play a role in phenol degradation in wastewater sludge (Basile and Erijman 2008; Tan 2021). The same technique may be applied to uncover the genetic potential for phenol degradation harnessed in soil.

In this study, we aim to explore the diversity of genes encoding phenol hydroxylase, a key initial enzyme

involved in phenol degradation, among the bacterial population in fuel-contaminated soil. This may lead to the discovery of novel functional genes which can then be applied to further improve various bioremediation-based approaches to treat phenol-contaminated environment.

## MATERIALS AND METHODS

### Sample collection and genomic DNA extraction

Silt soil sample was obtained surrounding a commercial gas station in Central Jakarta, Indonesia, at 20 cm depth from the surface. Total genomic DNA was extracted from soil using ZymoBIOMICS® DNA kit (Zymo Research). The quality and quantity of obtained DNA were determined using agarose gel electrophoresis and NanoDrop™ 2000 (ThermoFisher Scientific).

### Amplification of phenol hydroxylase-encoding gene

Partial phenol hydroxylase encoding gene was amplified with a pair of universal primers designed by Futamata et al. (2001): pheUf (5'-CCAGGSBGARAARG AGARGAARC-3') and pheUr (5'-CGGWARCCGCGC CAGAACCA-3') resulting in a 600-bp amplicon. The polymerase chain reaction (PCR) mix consisted of 10 pmol of each primer, 100 ng of DNA template, GoTaq® Green Master Mix (Promega), and nuclease-free water to a total volume of 50 µL. The cycling conditions were as follows: 10 minutes of pre-denaturation at 94°C, 30 cycles of 1 minute of denaturation at 94°C, 1 minute of annealing at 58°C, and 1 minute of extension at 72°C; followed by 10 minutes of final extension at 72°C. The PCR product was then visualized on a 1% $^{w}/_{v}$ agarose gel in 1x TAE buffer (pH 8.3) stained with ethidium bromide. Gels were visualized using UV transilluminator and the corresponding fragment (600bp) was excised from the gel and purified with QIAquick Gel Extraction Kit (QIAGEN).

### Library construction

Purified partial phenol hydroxylase fragments were cloned into *Escherichia coli* strain DH5α using the pGEM®-T Easy vector (Promega) according to the manufacturer's instruction. Each clone was assigned an individual code beginning with PJK followed by clone number. Clones carrying the insert were selected based on blue-white screening. Insert DNA was amplified from each clone using the M13f (5'-GTAAAACGACGGCCAGT-3') and M13r (5'-CAGGAAACAGCTATGAC-3') primer pair (Messing 1983). PCR reaction mixtures were as described above for the amplification of phenol hydroxylase genes. PCR conditions were as follow: 5 minutes of pre-denaturation at 95°C, 30 cycles of 30 seconds of denaturation at 95°C, 30 seconds of annealing at 50°C, and 90 seconds of extension at 72°C; and 5 minutes of post-extension at 72°C (Messing 1983).

### Sequencing and phylogenetic analysis

A total of 30 amplified clones were sent for sequencing at Macrogen, South Korea. The sequences were submitted to the GenBank database under accession number MN392977-MN393003 and compared to the GenBank database using BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was done by including chosen hits as references using ClustalW and neighbor-joining algorithm on MEGA7 with 100 bootstrap repetitions (Kumar et al. 2016).

## RESULTS AND DISCUSSION

### Results

Total DNA extracted from silt soil was presented as an intact band on agarose gel (data not shown). The total concentration was 101.5 ng $\mu L^{-1}$, with A260/280 of 2.1 and A260/230 of 1.9. Altogether these results indicate that the DNA extract is suitable for further analysis. Amplification with a primer pair targeting for partial phenol hydroxylase-encoding genes resulted in non-specific amplification, as seen by the presence of multiple bands upon gel visualization (Figure 1). As described by Futamata et al. (2001), the pheUf and pheUr primer pairs used in this study would generate a 600-bp band, and this was the brightest band observed on our agarose gel. The band was purified from the gel and cloned to pGEM®-T easy to generate 30 clones.

BLASTX analysis on the clones revealed the presence of 11 different hits, all of which were 86-99% similar to proteins associated with phenol degradation, including the largest subunit of multicomponent phenol hydroxylase, soluble diiron monooxygenase, phenol hydroxylase large subunit, and YHS domain-containing protein (Table 1). A majority of the clones (87%) showed the highest similarity with sequences belonging to uncultured bacterium (Table 1). Therefore, representative bacterial sequences with known genus or species that showed the next highest similarity for respective clones were selected as reference sequences in the phylogenetic analysis, including that of *Acidovorax* sp. Root 219, *Paraburkholderia oxyphilic*, *Ralstonia* sp. clone MF1WH1, *Hydrogenophaga* sp. T4, and *Pseudoxanthoonas spadix* BD-a59 (Figure 2).

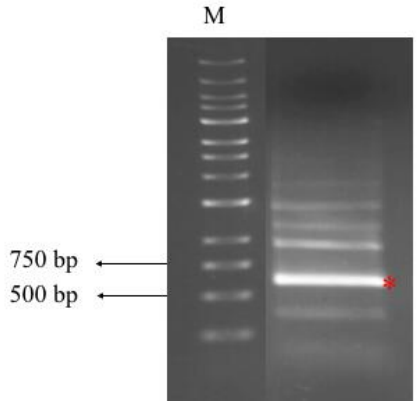


**Figure 1.** PCR amplification of partial phenol hydroxylase-encoding gene. Lane M: 1 kb DNA Ladder (Genesta™). Partial phenol hydroxylase-encoding gene is approximately 600 bp in size and is indicated with an asterisk

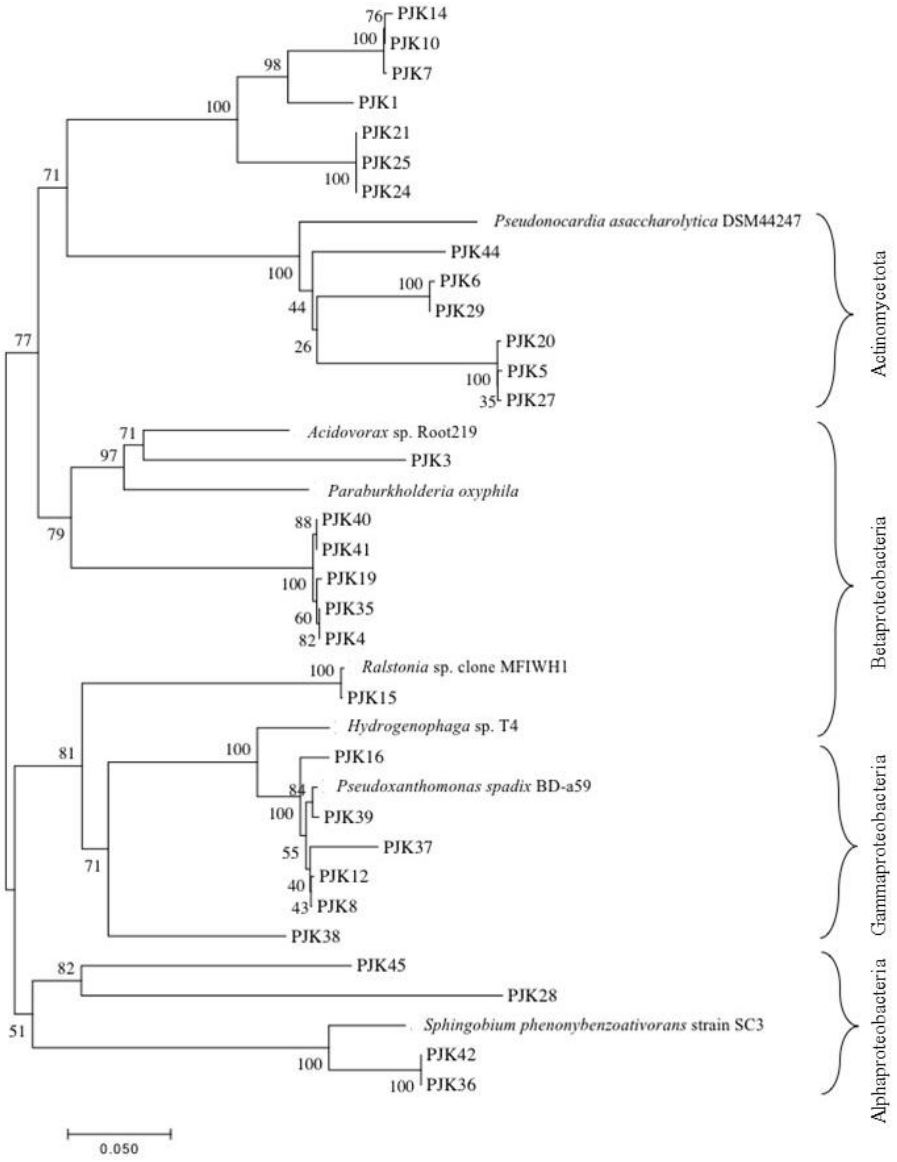


**Figure 2.** Phylogenetic tree of cloned partial phenol hydroxylase genes (PJK) and selected reference bacterial strains. Numbers at each branch represent bootstrap value with 100 repetitions. Bar scale on the left right indicates genetic distance

**Table 1.** Top hits from comparative analysis of recombinant clones through BLASTX

| Protein | Organism | Number of clone(s) | Identity (%) | Accession number | Clone number (PJK) |
|---|---|---|---|---|---|
| Largest subunit of multi-component phenol hydroxylase | Uncultured bacterium | 7 | 88-89 | BAF41005.1 | 1,7,10,14,21,24,25 |
| Soluble diiron monooxygenase | Uncultured bacterium | 1 | 86 | CCO97094.1 | 3 |
| | Uncultured bacterium | 5 | 95-97 | CCO97193.1 | 8, 12, 16, 37, 39 |
| | Uncultured bacterium | 1 | 98 | CCO97175.1 | 15 |
| | Uncultured bacterium | 1 | 90 | CCO97068.1 | 38 |
| | Uncultured bacterium | 4 | 87-88 | CCO97030.1 | 6, 27, 29, 44 |
| | Uncultured bacterium | 1 | 94 | CCO97057.1 | 28 |
| Phenol hydroxylase large subunit | Uncultured bacterium | 5 | 98-99 | SCB66043.1 | 4, 19, 35, 40, 41 |
| | Uncultured bacterium | 1 | 99 | SCB66008.1 | 45 |
| YHS domain-containing protein | *Pseudonocardia asaccharolytica* | 2 | 91-93 | WP_028929691.1 | 5, 20 |
| | *Sphingobium phenoxybenzoativorans* | 2 | 97 | WP_070156803.1 | 36, 42 |

Phylogenetic analysis showed that clones obtained in this study were clustered with the phenol hydroxylase genes of known aromatic hydrocarbon degraders belonging to Actinomycetota and Proteobacteriodota (Figure 2). Most clones (56.7%) were closely related to members of the phylum Proteobacteriodota, representing the Alpha-, Beta-, and Gammaproteobacteria classes.

**Discussion**

Partial phenol hydroxylase-encoding genes present within soil sample were amplified with a pair of degenerate primer designed by comparing amino acid sequences of phenol hydroxylase in six different bacterial species. This revealed the presence of a conserved region present in the six samples which can thus be used to partially amplify the gene of interest (Futamata et al. 2001). Upon visualization, multiple bands were observed on the agarose gel (Figure 1). The use of degenerate primers may be responsible for non-specific amplification. These oligonucleotides contain a population of primers wherein, during synthesis, alternative bases are appended at one or more positions. The use of degenerate primers may increase sequence coverage, and is especially useful to amplify a gene from different organisms; however, this may also lead to unspecific amplification (Hugerth et al. 2014).

Analysis of partial phenol hydroxylase-encoding genes using BLASTX showed that the cloned fragments were closely related to 11 hits that encompasses 4 different proteins- largest subunit of multicomponent phenol hydroxylase, soluble diiron monooxygenase, phenol hydroxylase large subunit and YHS-domain containing protein, all of which are associated with phenol degradation (Table 1). Phenol hydroxylases can be categorized into the single component and multicomponent enzyme group, of which the latter is more commonly found in the environment (Silva et al. 2013). The largest subunit of multicomponent phenol hydroxylase (LmPH) harbors the catalytic domain for phenol degradation, as seen in *Pseudomonas* sp. strain CF600 (Futamata et al. 2001). Soluble diiron monooxygenases are a group of multicomponent enzymes with a diiron center within its active site. These enzymes are able to catabolize and detoxify various aromatic substrates, including phenol, by catalyzing the initial hydroxylation step (Petkevičius et al. 2019). The YHS-domain is a 50-amino acid long region named after the three most conserved amino acid residues, tyrosine (Y), histidine (H), and serine (S) (Khor 2014). This domain, often present in ATPase and multicomponent phenol hydroxylase, is predicted to bind copper ions; however, further investigation is required to support this hypothesis (Linder 2015).

Phylogenetic analysis revealed the presence of 5 major clusters, with a majority (56.7%) of the clones obtained being closely related to Proteobacteriodota, specifically Alpha-, Beta-, and Gammaproteobacteria. A smaller cluster comprised of 6 clones (20.0%) was associated with the soil dweller Actinomycetota, while 23.3% of the clones were not clustered with any reference bacterium. This indicated a wider range of phenol hydroxylase gene diversity in soil compared to that reported in other studies. Futamata et al. (2001) reported that the phenol hydroxylase gene population in aquifer soil contaminated with the chlorinated hydrocarbon trichloroethylene was mainly composed of Betaproteobacteria and Gammaproteobacteria. In addition, phenol hydroxylase (*pheN*) gene detection on bacterial isolates recovered from oil-contaminated soils showed that all of the isolates harboring this gene belonged to Alpha- and Gammaproteobacteria (Sun et al. 2018). Representing a different habitat, our previous study also demonstrated that Betaproteobacteria dominated the phenol hydroxylase gene composition in the aeration and microbial recovery tank of a wastewater processing plant (Tan et al. 2021).

Clone PJK 36 and PJK 42 are both closely associated with *Sphingobium phenoxybenzoativoran* strain SC3 - an Alphaproteobacteria (Figure 2). This bacterium utilizes diphenyl ether (DE) and 2-carboxy-DE commonly used in the agricultural, pharmaceutical, and chemical industries, as its sole carbon and energy source (Cai et al. 2017). The presence of a diaryl ether linkage is responsible for its persistence in the environment. Degradation of DE and 2-carboxy-DE is solely catalyzed by a newly discovered diphenyl ether dioxygenase which is far more efficient than previous pathways requiring two different dioxygenases and one dehydrogenase (Cai et al. 2017).

Seven of the obtained clones are closely related to *Paraburkholderia oxyphila* (PJK3), *Acidovorax* sp. Root219 (PJK4, PJK19, PJK 35, PJK40, and PJK 41), and *Ralstonia* sp. clone MFIWH1 (PJK 15), all of which are members of Betaproteobacteria. *P. oxyphila* was isolated from forest soil in Japan (Otsuka et al. 2011), whereas *Acidovorax* sp. Root219 was isolated from around the roots of *Arabidopsis thaliana* (Bai et al. 2015). Aromatic hydrocarbons are ubiquitous in soil environment, where nutrients are often scarce, thus imposing a selection pressure against microbes to utilize them as its carbon source. *P. oxyphila* is also known to catabolize the naturally occurring aromatic compound catechin and its derivatives including, *p*-hydroxybenzoic acid and vanillic acid (Otsuka et al. 2011).

Clone PJK28, PJK36, PJK42, PJK45 clustered around *Pseudoxanthomonas spadix* BD-a59, member of the class Gammaproteobacteria. This bacterium was isolated from gasoline-contaminated soil and was able to degrade benzene, toluene, ethylbenzene, xylene (BTEX), all of which are major aromatic hydrocarbons in fossil fuel products. These compounds are listed as priority pollutant by the US Environment Protection Agency (USEPA) and, due to their relatively high solubility, are dominant contaminants in soil and groundwater (Choi et al. 2012).

The phylum Actinomycetota comprises of Gram-positive bacteria that can be found in the terrestrial and aquatic environment, though members of this phylum predominantly reside within the soil environment (Barka et al. 2016). They are able to catabolize various carbon sources, including phenol (Nogina et al. 2020). One of the reference species, *Streptomyces setonii* strain ATCC 39116, was reported to degrade aromatic hydrocarbons such as phenol and benzoate (Barton et al. 2018). *Pseudonocardia asaccharolytica* strain DSM 44247 was

isolated from tree bark compost and catabolizes dimethyl-disulphate (DMDS) as its sole carbon source (Reichert et al. 1998). Though DMDS is an aliphatic hydrocarbon, results obtained in this study indicate the presence of phenol hydroxylase gene in this species, which may indicate its ability to degrade phenol and possibly other aromatic hydrocarbons.

Hydroxylases, also known as oxygenases, recognize a wide variety of aromatic substrates (Tan and Parales 2016). Therefore, despite that the bacterial references showing similarity to the phenol hydroxylase genes in this study were reported for their ability to degrade other aromatic compounds, they may be capable of utilizing phenol as its sole energy source as well (Nešvera et al. 2015). Although not all bacterial hits have been directly linked to phenol degradation capability in previous reports, it is possible that the reference bacteria possess a gene that encodes for related monooxygenases for the degradation of other structurally similar aromatic compounds.

Interestingly, one cluster was separated from all reference sequences and clones. This indicates that members of this cluster have possibly never been reported before and they belong to a novel cluster. It would be interesting to study the complete sequence of this gene variant and predict its function in respect to aromatic compound catabolism. This could be done by designing primers that are move outward this sequence to perform bidirectional genome walking to obtain full sequences and further characterize genes present in this cluster.

This study illustrates a plethora of bacterial groups that may be responsible in phenol degradation. Such information may be put into consideration in phenol contamination treatment, either at the laboratory or industrial scale, in which treatment conditions that favor the growth of prominent pollutant degraders may be employed to increase the efficiency and effectiveness of the treatment. Clones obtained in this study belong to Proteobacteriodota and Actinomycetota, with more clones related to the prior. Interestingly, seven of the obtained clones clustered away from the other clones and bacterial references used, which may indicate the presence of a novel gene variant. Information gathered in this study may be helpful in developing a bioremediation-based approach as an alternative to conventional techniques in treating phenol-contaminated environment.

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