

## Short Communication: Preliminary phylogenetic analysis of bacteria producing laccase isolated from Gunung Pancar, Bogor, Indonesia

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**Abstract.** Mar WW, Rohman A, Muwafiqi NH, Laras GA, Agustina D, Asmarani O, Puspaningsih NNT. 2020. Short Communication: Preliminary phylogenetic analysis of bacteria producing laccase isolated from Gunung Pancar, Bogor, Indonesia. *Biodiversitas* 21: 2113-2118. Interpretation of phylogenetic trees is essential in understanding relationships between organisms, as well as their characteristics, bionetwork, and even their genomic and developmental ecology. The aim of this research is to analyze the phylogenetic bacteria producing laccase isolated from Gunung Pancar Bogor, Indonesia. Phylogenetic analysis of *Geobacillus kaustophilus* TP-02 producing laccase was performed using alignments with all *Bacillus* clusters. A phylogenetic tree was constructed by the neighbor-joining (NJ) method, using the maximum likelihood parameter. Laccase was purified using ammonium sulfate precipitation to 2.67-fold. The activity of crude laccase was determined to be 93.39 U/ml using 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as the substrate. Moreover, *Geobacillus* related to counting some *Geobacillus* genus such as *Geobacillus thermocatenulatus*, *Geobacillus* sp. MAS1, *Geobacillus thermoleovorans*, and *Geobacillus zalihae*, having similarity under 91% with comparison to the *Geobacillus kaustophilus* sequence in GenBank. Therefore, results show that *G. kaustophilus* TP-02 does not have any closeness with other *Geobacillus* genera, even though these species have the same ancestor.

**Keywords:** *Geobacillus kaustophilus* TP-02, laccase, neighbor-joining method, phylogenetic tree

**Abbreviations:** ABTS: 2,2-diazino-bis (3-ethylbenzothiazoline-6-sulfonic acid), NJ: neighbor-joining method, LB: Luria-Bertani, MEGA X: Molecular Evolutionary Genetics Analysis, NCBI: National Center for Biotechnology Information, 16S rDNA: 16 Svedberg Ribosomal-Deoxyribonucleic Acid

### INTRODUCTION

Laccases (EC 1.10.3.2, benzenediol: oxygen oxidoreductases) are multicopper containing enzymes that oxidize a variety of substrates, such as mono-, di- and polyphenols, aromatic amine, and other non-phenolic compounds, along with reduction of dioxygen to water (Moon et al. 2018). These enzymes are stable enzymes with low levels of substrate specificity, making them suitable choices for biotechnological and industrial applications (Siroosi et al. 2016). Such applications contain decontamination of manufacturing wastes, frequently from paper and pulp, fabric, and petrochemical industries, the construction of biosensors for the detection of phenolic pollutants (Younes and Sayadi 2011) pharmaceutical and cosmetic industries (Ricklefs et al. 2014). Laccase can be explored by thermophilic bacteria.

Recently, production of thermostable laccase was received by exploration of thermophilic bacterial strain (Sharma et al. 2019). These are categorized into numerous classes based on their temperature ranges for microbial

growth (Nwokorie 2014), and microorganisms may favor diverse ranges of temperatures. Therefore, microorganisms are considered as psychrophiles, mesophiles, thermophiles, and hyperthermophiles. Among them, thermophilic organisms are important organisms for microbiologists and biochemists due to their commercial applications (Baltaci et al. 2017). Furthermore, microorganisms that live under conditions such as hot springs, volcanic, and geothermal regions have exclusive features used for industrial applications. Laccase can be produced by thermophilic bacteria such as *G. kaustophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. zalihae*, *Geobacillus* sp. MAS1, *Bacillus mycoides*, and *Bacillus anthracis*. Thus, scientists are targeting learning towards thermophilic bacteria as a potential source for thermostable industrial enzymes (Nwokorie 2014). In addition, several methods have been accepted to categorize laccase sequences. One of the most common methods is phylogenetic analysis, which is a sequence-based clustering method (Hoegger et al. 2006) by Molecular Evolutionary Genetics Analysis (MEGA X) (Kumar et al. 2018).

Phylogenetic analysis is one of the most commonly used methods in systematics to understand the diversity of living things through reconstruction of relationships (Harnelly et al. 2018). Along with rapid advancement of molecular biology, data of sequences have been used in many phylogenetic studies to attain more exact information (Lestari et al. 2018). The common methods of protein-based phylogeny are founded on multiple alignments of enzyme sequences and counting of distances (insertions, removals, and mutations) between these sequences. The distance matrix of the suitable clustering system is used to create the phylogenetic tree. Mostly, phylogenetic analysis of enzyme sequences is a powerful instrument for group and interpretation of the taxa (Satpathy 2014). Surely, phylogenetic tree interpretation is constructed for most of the comparative evolutionary biology. A road map is an inferred pattern of branching to understanding any other hierarchy of characters inclined by those groups. Such characters can be relatively evaluated by ascending into the spectrum of ancestor-descendant relationships within groups and their subgroups (Staton 2015). Furthermore, with even a basic understanding of general principles and conventions, researchers can likely attain valuable information about the beginning, evolution, and probable function of phylogenetic tree proteins (Mallika et al. 2011). Therefore, phylogenetic trees help construct the relationships between organisms and approximate differences that happen from one ancestor to the offspring (Dharmayanti 2011).

In the present study, we attempt to extend the research towards production of extracellular laccase from investigation of thermophilic bacterial strains. These strains were isolated from Gunung Pancar Hot Spring, Bogor, Indonesia. The laccase was produced from *G. kaustophilus* TP-02 and purified partially using ammonium sulfate precipitation. In addition, phylogenetic analysis of *G. kaustophilus* TP-02 producing laccase was performed using alignments with all *Bacillus* clusters. Therefore, the ability of thermophile laccase has been reported by phylogenetic tree interpretation.

## MATERIALS AND METHODS

### Isolation and screening of laccase producing bacteria

The samples were collected from Gunung Pancar Hot Spring, Bogor, Indonesia. The obtained samples (temperature 80°C) were conserved at 4°C until further use. After incubation, the samples were enlarged by incubating on Luria-Bertani (LB) agar medium plates at 60°C for 18 h. The enlarged samples were streaked on LB agar medium and then incubated at 60°C for 18 h. The diverse bacterial colonies were separately streaked and purified several times based on color and morphology. Subsequently, the gained bacterial pure cultures were deposited at 4°C until investigation.

Laccase activity was screened by isolating the thermophilic bacterial used guaiacol 0.1% as a substrate. These isolated bacteria were marked by adding guaiacol 0.1% onto LB agar plates and incubated at 60°C for 24 h. The occurrence of reddish-brown color indicated isolated species' ability to produce laccase. For confirmation, the selected bacteria was rechecked for laccase activity by using cofactor of CuSO<sub>4</sub> 0.1 mM on LB media both with guaiacol and without guaiacol.

### Laccase activity

Laccase activity was analyzed by measuring the growth with 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as the substrate in absorbance at 420 nm for 5 min (Zheng et al. 2017). A 900 µl of reaction mixture included 300 µl of enzyme and 600 µl of 0.4 mM ABTS. Enzyme assay was carried out by monitoring the absorbance increase at 420 nm ( $\epsilon_{420} = 36,000 \text{ M}^{-1} \text{ cm}^{-1}$ ). The amount of enzyme was defined as one unit of enzyme activity that oxidized 1 µmol of substrate per min (Wang et al. 2010). Protein concentration was assessed via the Bradford method using bovine serum albumin as the standard (Ben Younes and Sayadi 2011). All examinations were performed in triplicate.

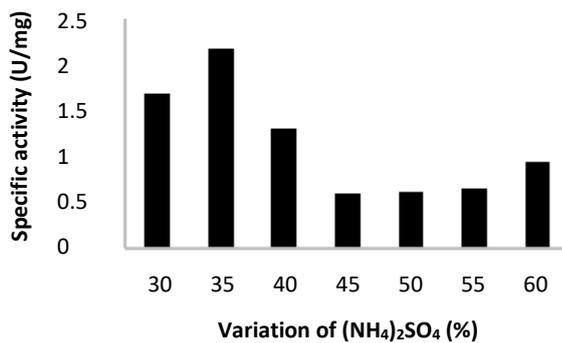
### Partial purification of thermophilic laccase

Purification steps were exposed by crude enzyme, which consisted of concentrating proteins using ammonium sulfate purification (Agrawal et al. 2019). Specifically, 100 mL of crude enzyme solution including proteins was fractionally precipitated by ammonium sulfate solution at 4°C to reach a final concentration ranging from 30 to 85% (w/v). During this procedure, ammonium sulfate was gradually added to the crude enzyme with gentle stirring. Precipitation was achieved over 12 h at 4°C. The enzyme was improved by centrifugation at 12,000 rpm for 15 minutes and then dissolved in 100 mM citrate-phosphate buffer (pH 4.0) (Zheng et al. 2017).

### Phylogenetic analysis

Nucleotide sequences of laccases were collected using GenBank (NCBI) with a FASTA arrangement file. Nucleotide alignments were carried out using the MEGA X program with amino acid substitution type (Jones et al. 1992). This analysis included 57 sequences of laccase from different *Geobacillus* strains. The maximum likelihood method and Jones-Taylor Thornton (JTT) matrix-based model was used to infer ancestral state. The phylogenetic tree shows a set of selected amino acids at each ancestral node based on their inferred likelihood per site. Evolutionary diversity analyses were also conducted using the MEGA X program (Kumar et al. 2018). Nucleotide sequences alignment parts of *G. kaustophilus* are shown in Figure 1.





**Figure 3.** The specific laccase activity was determined after addition of various percentage concentrations of saturated ammonium sulfate (30-85%)

### Laccase activity and partial purification

The crude laccase activity was measured to be 93.39 U/ml. Furthermore, the specific activity was recorded as 0.819 U/mg of extracellular crude laccase. According to results, the purification level is listed in Table 1. The purification step showed that the specific laccase activity was increased after optimum ammonium sulfate addition (35%), it indicated that the other protein contaminant was decreased from 114 mg/mL to 0.488 mg/mL.

In this situation, protein concentration of crude enzymes was performed by about 114 mg/mL. Ammonium sulfate fractional precipitation is an easy and low-cost method and was completed for partial protein purification of crude laccase. Thereafter, various ammonium sulfate concentrations were estimated for highest laccase precipitation, and the highest precipitation was found at 35% (Figure 3). In this stage, the enzyme was reached with 2.67 times purification and 1.14% yield (Table 1).

### Phylogenetic analysis

In the present study, nucleotide sequences of laccase were attained from GenBank (NCBI) with FASTA format file. A phylogenetic tree was created with the neighbor-joining method, using the maximum likelihood parameter and JTT matrix-based model. In total, 57 sequences from different *Geobacillus* strains were designated for this

present study. A total of 57 sequences were observed to be reliable for phylogenetic analysis as well as for a data mining approach. The complete alignment of these sequences with a Clustal-X instrument was combined using the MEGA X software. The phylogenetic tree showed a taxonomic clustering through the major taxa (Satpathy 2014). The neighboring relationship of sequences was demonstrated by constructing a phylogenetic tree (Figure 4).

The laccase sequence of the fragments has been described by calculating the percent identity with gene from *Geobacillus* genus (Table 2). The phylogenetic relationship exhibited that *G. thermoleovorans* and *G. zalihae* were closely correlated to *G. kaustophilus*, which varied about 10% identity, while *G. thermocatenulatus* and *G. thermocatenulatus* were strictly correlated to *G. kaustophilus*, with sequence variance as much as 9%. Furthermore, *G. genomosp. 3*, *G. lituanicus*, *G. stearothermophilus*, *G. uzenensis*, *G. subterraneus*, *G. stearothermophilus* ATTC 12980, *Geobacillus thermodenitrificans*, and *G. subterraneus* have been identified to *G. kaustophilus* with more than 10% differences, while *G. galactosidasius* and *Bacillus amyloliquefaciens* have differed nearly about 50% from *G. kaustophilus*.

**Table 2.** Comparison of identity (%) some laccase sequence members of the *G. kaustophilus* family (Altschul et al. 2005)

Query	Taxonomy affinity	Percent identity
11739	<i>Geobacillus kaustophilus</i>	100%
14175	<i>Geobacillus zalihae</i>	90%
129121	<i>Geobacillus thermoleovorans</i>	90%
180633	<i>Geobacillus genomosp.3</i>	87%
93381	<i>Geobacillus lituanicus</i>	85%
209139	<i>Geobacillus stearothermophilus</i>	85%
23615	<i>Geobacillus thermocatenulatus</i>	91%
197633	<i>Geobacillus uzenensis</i>	84%
127911	<i>Geobacillus thermocatenulatus</i> (OXB86401)	91%
7253	<i>Geobacillus subterraneus</i>	84%
100303	<i>Geobacillus stearothermophilus</i> ATTC 12980	85%
30911	<i>Geobacillus galactosidasius</i>	56%
165335	<i>Geobacillus thermodenitrificans</i>	85%

**Table 1.** The purification level

Purification step	Total volume (mL)	Total enzyme activity (U/mL)	Total protein activity (mg/mL)	Specific activity (U/mg)	Yields (%)	Purification (fold)
Crude enzyme	100	93.39	114	0.819	100	1
Ammonium sulfate fractional precipitation (35%)	0.4	1.066	0.488	2.184	1.14	2.67



comparable to those of *G. vulcani* DSM 13174T and *G. kaustophilus* HTA426 *rrnA* and *rrnB* (99.3-99.6% sequence similarity) (Kuisiene et al. 2007), whereas the sequence of *G. stearothermophilus* ATCC 12980 has been identified to have close relationship and comparison to *G. kaustophilus* (85% sequence similarity) in this study. In brief, laccase can be produced by *G. kaustophilus* TP-02 with the partial purification level of optimum ammonium sulfate 35%. In addition, laccase can also be aligned using alignment sequences of amino acid from thermophilic bacteria action. The potentially thermophilic bacteria were classified with the laccase alignment sequences and phylogenetic tree analysis. In addition, different nucleotide sequences were dispensed along the gene sequence, in the method of difference sequences, nucleotide substitutions, and deletions. *Geobacillus* were related to counting some *Geobacillus* genus, such as *G. thermocatenulatus*, *Geobacillus sp.* MAS1, *G. thermoleovorans*, and *G. zalihae* having similarity under 91% with comparison of *G. kaustophilus* sequence in GenBank. Therefore, results show that *G. kaustophilus* does not have any closeness with another *Geobacillus* genus, even though these species have the same ancestor. This research assumed that the thermophile of *G.kaustophilus* TP-02 isolated from Gunung Pancar Hot Spring, Bogor, Indonesia as novel strain bacteria producing laccase.

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