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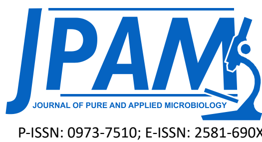

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RESEARCH ARTICLE OPEN ACCESS

Isolation, Screening and Identification of Lignin Degraders from the Gut of Termites *Odontotermes obesus*

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Abstract

The mounting disquiets about the usage of precarious chemicals in the textile industry have steered to the development of eco-friendly and biological methods of fiber processing in the ever-escalating horizon of textile fibers. The current study targets the isolation, identification, and screening of lignin-degrading bacteria from termite gut microflora which could be employed in the textile trade, especially in coir industries for developing a biological method for softening coir fibers. Based on the morphology and taxonomic keys, termites used in the study were identified as *Odontotermes obesus*. The bacteria isolated from the termite gut having lignolytic activity were picked by using the methylene blue dye decolorizing method. The same was confirmed by using tannic acid. The isolates were then identified as *Kosakonia oryzendophytica* and *Pseudomonas chengduensis* by 16s rRNA sequencing. The isolates were further checked for their ability to produce extracellular lignolytic enzymes. The enzyme concentration was found to be significantly higher in the medium containing the microbial consortium than in those with the individual cultures. The consortium filtrate has MnP activity of 41.6 U/mL, LiP activity of 114.3 U/mL, and laccase activity of 61.85 U/mL at 72 hours of incubation. It was found that the enzyme activities were increased considerably until 72 hours of incubation but showed an insignificant increase at 96 hours.

Keywords: Lignolytic, Peroxidases, Laccase, Facultative Anaerobe

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INTRODUCTION

Lignocellulose is a most profuse and low-cost renewable biological resource and signifies a chief source of renewable biotic material, on the other hand it is challenging to be degraded due to its multifaceted assembly.[1] The sale and export of lignocellulosic fibres contribute significantly to the income and food security of poor farmers and those working in fibre processing and marketing in many developing nations.[2] The physical parameters and characteristics of natural lignocellulosic fibers hinder the processing methods. The outsized and variable diameter, high microfibrillar angle, hemicellulose and greater lignin content have been found responsible for its poor power-driven reinforcement. Chemical treatments have many disadvantages as it releases harmful compounds to the environment, and may deteriorate the strength of natural fibers with time.[3] Researches are underway to discover eco-friendly approaches to attain softened natural fibers by removing lignin which plays the foremost role in its tough nature. The ability of gut bacteria in termites to disrupt the plant cell wall polysaccharide is closely 30% - 40% superior to the large herbivores. Nonetheless, limited studies have been performed on the identification and screening of lignolytic bacteria from the gut of termites.

The most important lignolytic enzymes are laccases (E.C. 1.10.3.2) and peroxidases: manganese peroxidase (E.C. 1.11.1.13) and lignin peroxidase (E.C. 1.11.1.14).[4] A diverse array of microbes particularly, fungi and bacteria, including actinomycetes, are capable to decompose lignin, however, fungi have the highest degrading ability.[5]

The termites are one of the greatest significant lignocellulose decomposers in the natural biota predominantly in the tropics. Termites, which are proficient lignocellulose degraders, flourish on plant debris and contribute to carbon mineralization, particularly in tropical and subtropical regions.[6-9] The gut microbial community comprises all three domains of organisms: Bacteria, Archaea, and Eukaryotes (protists). Fairly little is found in the foregut and midgut, whereas abundant microbiota is found in the hindgut.[10,11] There are several types of bacteria with diverse utilities in the termite's gut that have been isolated and identified. They were hemicellulose degrading bacteria[12] lignolytic bacteria,[13] cellulolytic bacteria,[14] aromatics degrading bacteria,[15] and nitrogen-fixing bacteria.[16] The most plentiful bacteria which have been recognized from both higher and lower termites belong to the species of strict or facultative anaerobes. The role of the facultative anaerobes may be to scavenge the oxygen that can infuse into the gut, effectually keeping the gut anaerobic.[17] The present study aims at isolation, identification, and preliminary screening of lignin-degrading microbes from the gut of termites, *Odontotermes obesus*.

MATERIALS AND METHODS

Collection and Identification of Termites

Soldier termites were collected from their nest from the backyard of the Central Coir Research Institute and were checked for their identity with the help of Trinocular Stereo Zoom Microscope (dissection microscope), LSZM1. The Identification of termites was done following the schemes proposed by Roonwal and Chhotani[18] and Chhotani.[19]

Bacterial Isolation

Worker termites were collected from their nests from the backyard of the Central Coir Research Institute campus and surface sterilized using 70% ethanol followed by mashing with mortar and pestle by adding one mL normal saline and subjected to serial dilution. The ground mixture was transferred to 99 ml Berg minimal media containing the following ingredients per litre: 2 g $NaNO_3$, 0.5 g K_2HPO_4, 0.02 g $MgSO_4{:}7H_2O$, 0.02 g $MnSO_4{:}7H_2O$, 0.02 g $FeSO_4{:}7H_2O$, and 0.5 g $CaCl_2{:}2H_2O$ with 1% kraft lignin as the sole carbon source and subjected to serial dilution. Nutrient agar plates were prepared with the addition of 1% kraft lignin as sole source of carbon and 25 mg/L methylene blue as an indicator dye for picking out the lignolytic microbes exhibiting zone of decolourization around the bacterial colonies.[20,21] Plates were inoculated with dilutions of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} respectively, and incubated at 37°C for 48 hours. The colonies with clearance zone were isolated and streaked over fresh plates and incubated at 37°C for 48 hours. The lignolytic activity was further confirmed

by using tannic acid in the culture medium as a substitute for lignin.[22]

Identification of the Isolated Bacterial Strains

The isolated DNA sample was amplified by performing PCR using 16S Forward (AGAGTTTGATCCTGGCTCAG) and 16S Reverse (GGTTACCTTGTTACGACTT) primers. 50 µl PCR mixture consisted of 1 µl template DNA, 5 µl *Taq* reaction buffer (10X), 1 µl 200 µM dNTPs, 0.25 µl of *Taq* DNA polymerase, 1 µl of 10 µM concentration of forward and reverse primers. The reaction mixture was finally made upto 50 µl with nuclease free water. The cycling conditions were; Initial denaturation at 95°C for 30 seconds, 30 cycles consisting of template denaturation at 95°C for 15-30 seconds, primer annealing at 45-68°C for 15-60 seconds, primer extension at 68°C for one minute per kb and a final extension at 72°C for 5 minutes. The amplicon was then electrophoresed in 2% SYBR SAFE agarose gel at 85 volts and visualized under UV and the concentration of the same was checked using Nanodrop ND 8000. The amplicon was further purified with the help of PureLink purification column (Invitrogen) and sequencing was done by sanger sequencing method by using forward and reverse primers in the ABI 3730xl cycle sequencer. Forward and reverse sequences were assembled and contig was generated after trimming the low-quality bases. The sequence analysis was performed using BLAST, the Bioinformatic tool of NCBI. Based on the maximum identity score first, a few sequences were nominated and aligned by multiple sequence alignment software ClustalO. The dendrogram was constructed. All sequences obtained were submitted to the GenBank nucleotide database (GenBank Accn No. OM943747, OM943748).

Enzymatic Assays

To perform the lignolytic enzyme assays, 5 mL of the culture media was drawn out at 24, 48, 72, and 96 hours respectively. The drawn cultures were then centrifuged at 12000 rpm to obtain the extracellular enzymes present in the medium. The supernatant (crude enzyme) obtained after centrifugation was taken for testing the presence of lignolytic enzymes produced by the microbes.

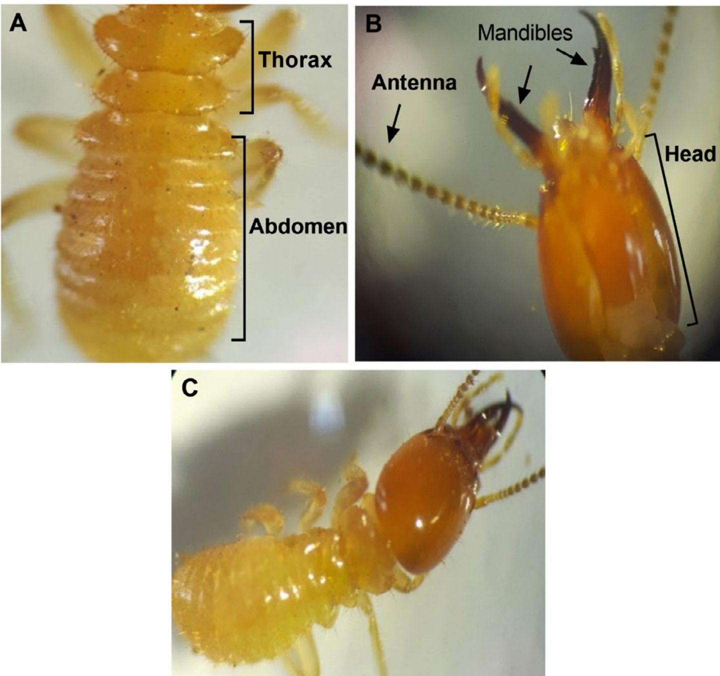

Figure 1. *Odontotermes obesus* under the stereomicroscope. A) Morphology of Thorax and abdomen. B) Head region and associated parts. C) Depiction of whole termite.

Lignin Peroxidase (E.C. 1.11.1.14) Assay

Lignin peroxidase enzyme activity was determined by observing the oxidation of azure B dye in the presence of H_2O_2 (100µM). The reaction mixture taken for the test comprised 125 mM of 1 mL sodium tartarates buffer (pH 3.0), 500 of 2 mM H_2O_2, 500 µL of 160 µM Azure B and 0.5 mL of the enzyme extract. The absorbance was read at 310 nm after the reaction is being initiated by the addition of 0.5 mL of H_2O_2. One unit of enzyme activity is equivalent to an decrease in absorbance of 0.1 units per minute per mL.[23]

Manganese Peroxidase (E.C. 1.11.1.13) Assay

Manganese peroxidase assay using phenol red was performed in the study. Five mL of reaction mixture was prepared with 1.0 mL sodium succinate buffer (50 mM, pH 4.5), 1.0 mL sodium Lactate (50 mM, pH 5.0), 0.4 mL manganese sulphate (0.1 mM), 0.7 mL phenol Red (0.1 mM), 0.4 mL H_2O_2 (50µM), gelatin 1 mg/mL and 0.5 mL of the enzyme extract. The reaction conducted at 30˚C was commenced by adding H_2O_2 . One mL of the reaction mixture was added with 40µL of 5 N NaOH and absorbance was read at 610 nm. The enzyme concentration was defined in such a way that one unit of the enzyme is equivalent to an absorbance increase of 0.1units /min/ml.[23]

Laccase (E.C. 1.10.3.2) Assay

Laccase activity was checked by ABTS method where 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) acts as substrate and the absorbance were measured at 420 nm. The reaction mixture used in the method contained 100 mM sodium acetate buffer, pH 5, 0.5 mM ABTS and 400 µl enzyme supernatant. An extinction coefficient of 36,000 $M^{-1}cm^{-1}$ for ABTS was used for calculating the enzymeactivity. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 µmol of substrate per minute.[24-26]

Statistical Analyses

Two-way ANOVA was performed using IBM SPSS v 20 (IBM Corporation, Armonk, NY) to analyze the significant difference (Tukey test, LSD; $P < 0.05$) in laccase, lignin peroxidase and manganese peroxidase assays results among the samples (*P. chengduensis, K. oryzendophytica* and *Consortium*) tested at different hours (24, 48, 72 and 96).

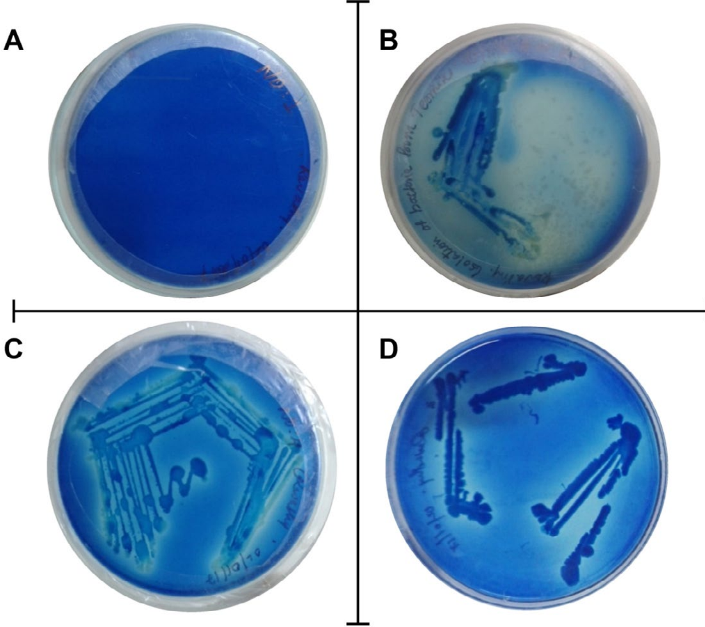

Figure 2. Petri dish tests of oxidation of methylene blue dye. A) Control plate, B) Plates showing the oxidation of methylene blue dye by the streaked isolate, C) Isolate 1(T1), D) Isolate 2 (S).

RESULTS AND DISCUSSION

Termite species differ in their rudimentary biology and ecology, including colony size, feeding, nesting, swarming, and reproductive behavior. Identification of termite species is a thought-provoking chore because of the vagueness in their morphological characteristics, the difficulty to distinguish morphologically distinct castes such as soldiers and alates, topographical changes, and the absence of overall methodical research for the identification of termite genera.[27] The identification based on soldier morphology is considered the finest route so far compared to alates owing to seasonal existences that prevent concurrent collections and portrayal of the castes.[28,29] Based on the morphological features observed under the stereomicroscope, the termites were identified as *odontotermes obesus* (Rambur) (Figure 1).

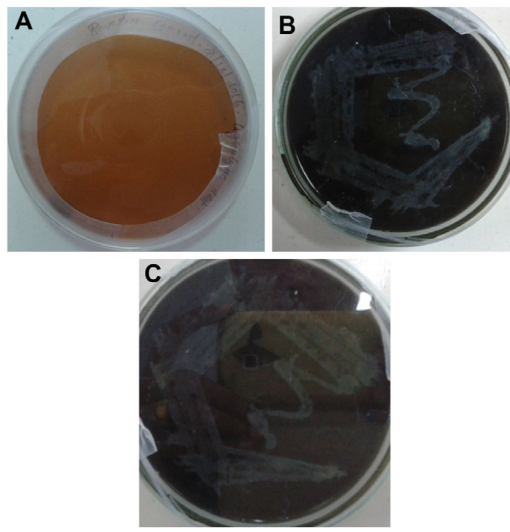

Figure 3. Plates showing Confirmatory test for lignolytic activity using tannic acid as a substitute for lignin. A) Control, B). Isolate 1(T1), C). Isolate 2 (S).

Distinct bacterial colonies with dye decolorization were observed on plates with 10^{-6} dilution (Figure 2). Decolorisation of methylene blue was used as a tool to identify and pick out the microorganism having lignolytic potential. In dye decolorization, the azo linkage is split either by reduction, the reaction used by some bacterial consortia under anaerobic conditions, or by oxidation, the reaction used by lignolytic white-rot fungi. Researchers found that lignolytic enzymes such as lignin peroxidase, manganese

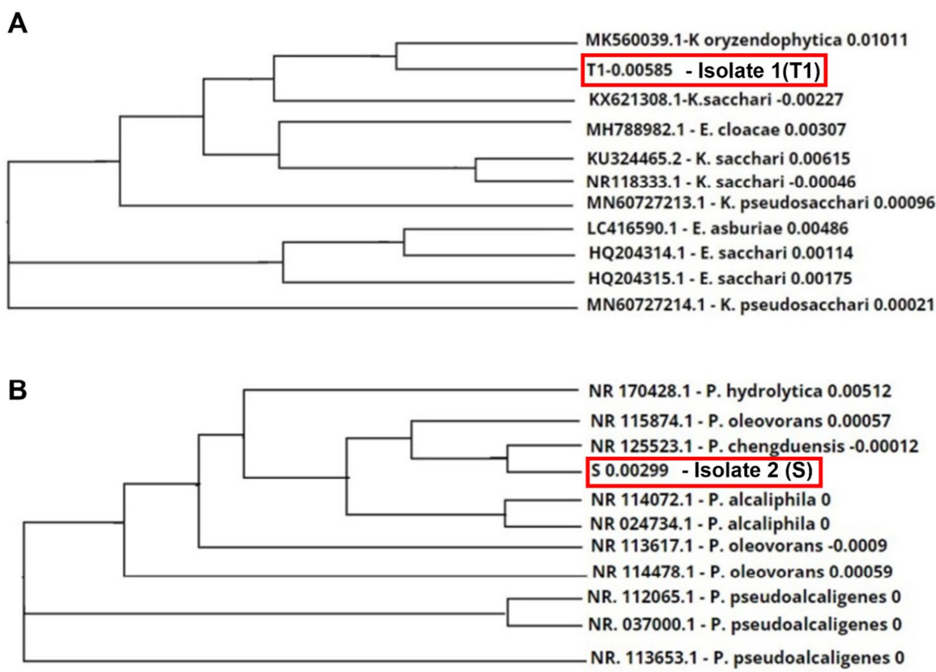

Figure 4. Dendrogram showing the 16s RNA sequencing and sequence similarities of the isolates with related genus. A) Isolate 1 (T1) *K. oryzendophytica*, and B) Isolate 2 (S) *P. chengduensis.*

peroxidase, and laccase were responsible for dye decolorization.[30] Previous scientific reports state that several bacteria are capable of dye decolorization, either in consortia or in pure cultures.[31]

All the isolates grown in a medium containing tannic acid showed color change which indicates the presence of lignin-degrading enzymes (Figure 3). The brown color of the medium might also be due to the action of extracellular phenoloxidases which resulted in the repolymerization of degradation products.[32,33]

Genetic ID employing 16S rRNA has been performed for more than a decade to investigate evolutionary connections among different living beings. The comparison of rRNA sequence aid to build a universal tree of life, separating all life on earth into three equidistant domains specifically Eukarya, Bacteria, and Archaea The microbial isolates that revealed lignin-degrading ability were identified as *Kosakonia oryzendophytica* (GenBank Accn No. OM943747) and *Pseudomonas chengduensis* (GenBank Accn No. OM943748). Based on the reports by Drancourt et al.,[34] when the resemblance percentage was ≥ 99% then the equivalent isolate was the same species, 97% to 99% can be specified that the analogous isolate represents the identical genus and the

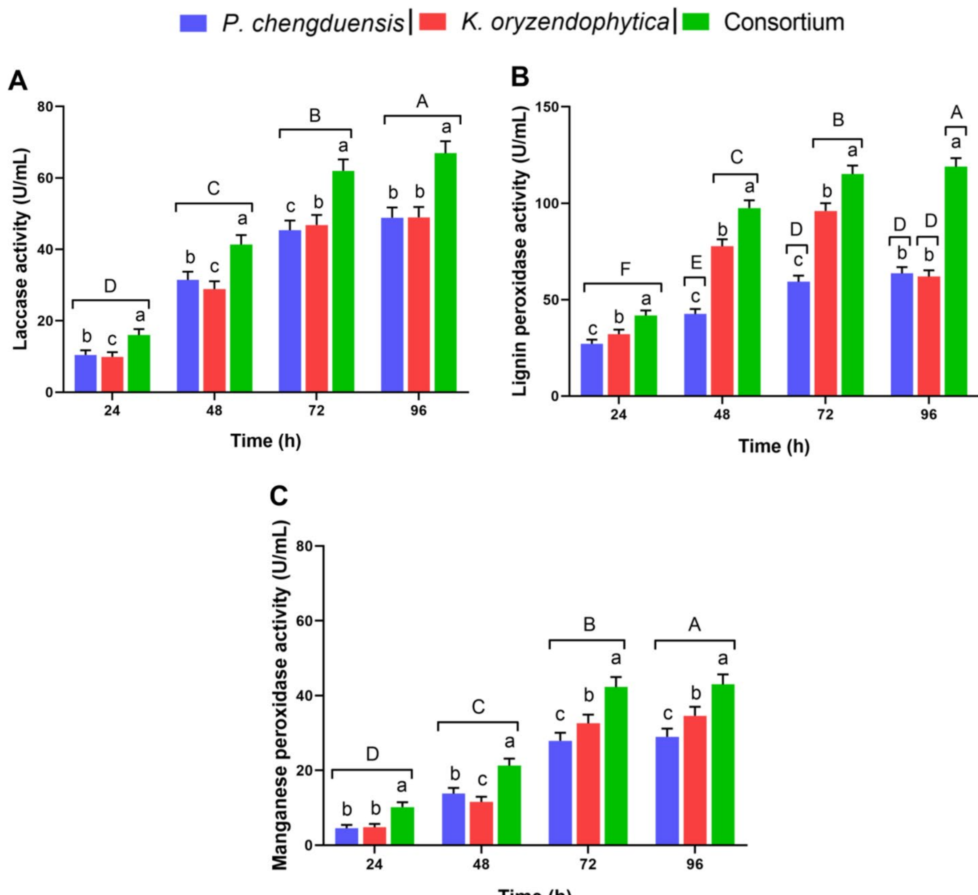

Figure 5. Laccase, lignin peroxidase and manganese peroxidase assays results of P. chengduensis, *K. oryzendophytica* and Consortium were tested at different hours. A) Laccase, B) Lignin peroxidase, and C) Manganese peroxidase activity (U/mL). Six replications (Mean±SE) were maintained for each activity. Vertical bars under different small letters were statistically significant, and the same letters indicate statistically insignificant (Tukey test, LSD; *P*<0.05) among the samples (*P. chengduensis, K. oryzendophytica* and Consortium). Different capital letters on the top of the vertical bar indicate that each sample tested at different hours (24, 48, 72 and 96) was statistically significant (Tukey test, LSD; *P*<0.05), same letters statistically insignificant.

resemblance percentage below 97% indicates that the isolate can be declared as a new species. The BLAST alignment results show that strain T1 exhibit a similarity of 99.57% with *Kosakonia oryzendophytica*, strain S had a similarity of 99.93% with *Pseudomonas chengduensis*, respectively. The dendrogram (phylogenetic tree) was built according to the comparison of 16S rRNA sequences of the isolated strains with those extracted from the GeneBank database (HTTP:// www.ncbi.nlm.nih.gov). Out of 16S rRNA sequences from various strains, ten strains with maximum homology were compared. The dendrogram was constructed by aligning all sequences with Clustal Omega. The dendrogram represented in Figure 4 verified the linkage of the isolates T1 and S each with the other ten strains. It was found that isolate T1 was closely related to *Kosakonia oryzendophytica* and isolate S was closely related to *Pseudomonas chengduensis* respectively.

Research has already proved the lignin-degrading ability of bacteria under the Enterobacter genus such as *E. cancerogenus, E. ludwigii* etc.[35] yet no studies have been done on *E. oryzendophyticus* (reclassified as *K. oryzendophytica*[36]) and *P. chengduensis.* Microbes have developed quite a lot of enzymes for mortifying the diverse constituents of lignocellulosic material. These enzymes comprise cellulases, xylanases, and lignolytic enzymes.[26] Among those, lignin peroxidase (LiP) and manganese peroxidase (MnP) have been predominantly responsible for lignin degradation.[23] Liew et al. reported that manganese peroxidase produced is far more superior as compared to lignin peroxidase, suggesting that MnP might be the prevailing enzyme involved in lignin deprivation.[37] A wide range of potential substrates has raised interest in the use of laccases in several industrial applications, such as delignification of pulp, bleaching of textile dye, detoxification of effluent, modification of biopolymers, and bioremediation.[38,39]

In the present study, all the bacterial isolates produced MnP, LiP, and laccase and showed a significant increase at 72 hours of incubation. The enzyme activity was found higher in the medium containing the microbial consortium than in those with the individual cultures. The consortium filtrate has MnP activity of 41.6 U/mL, LiP activity of 114.3 U/mL, and laccase activity of 61.85 U/m at 72 hours of incubation. It was found that both the enzyme activity was increased considerably until 72 hours of incubation but showed an insignificant increase at 96 hours. Hence, to attain the maximum changes in the shortest time, further analysis was performed with the optimum time of treatment as 72 hours.

The laccase, lignin peroxidase and manganese peroxidase assays results of *P. chengduensis, K. oryzendophytica* and Consortium were compared among the samples and the performance of individual sample at different hours (Figure 5). Overall, the *P. chengduensis* and *K. oryzendophytica* combined consortium results were highly significant within the samples and the individual sample activity at different hours (Figure 5). The individual treatments of *P. chengduensis* and *K. oryzendophytica* laccase and manganese peroxidase activities were increasing and highly significant at different hours (Figure 5a and c). On the other hand, lignin peroxidase activity of *P. chengduensis* and did not show a significant increase after 72 hours (Figure 5b). Conversely, *K. oryzendophytica's* lignin peroxidase activity was significantly reduced after 72 hours (Figure 5b).

CONCLUSION

Lignolytic enzymes have possible applications in various sectors such as chemical, food, fuel, paper, agricultural, textile, and other industries. Natural fibers are made of cellulose, lignin, and other constituents, of which lignin is responsible for the rigidity and hydrophobicity which in turn restricts the use of those tough fibers in various sectors of textile industries. The prominence of lignolytic bacteria has augmented since lignin-degrading bacteria have an eclectic forbearance for temperature changes, pH differences, and oxygen curb compared to fungi. The present study explored the incredible lignin-degrading capability of termites wherein the gut microflora are the real lignin biodegraders which helps the termites efficiently utilize the lignocellulosic matter. The bacteria isolated from the termite gut identified as *K. oryzendophytica* and *P. chengduensis* are found to have a strong potential for lignin degradation. Further investigations regarding the optimization

of growth conditions of the bacteria might improve the lignolytic activity of the isolated strains. Thus the finding will open its path to a superior substitute for the fundamental chemical practices which benefits the future textile trade to be more eco-friendly

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

The article does not contain any studies with human or animal participants performed by any of the authors.

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