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Production of Ligninolytic Enzymes by *Coptotermes curvignathus* Gut Bacteria

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***Abstract* – Maximum utilization of lignocellulosic biomass is contingent upon degrading the recalcitrant lignin polymer. Conventional methods employed in delignification require high inputs of energy and chemicals, resulting in the release of highly toxic effluents. The ability of gut flora of *Coptotermes curvignathus* in lignin degradation was investigated in this study. Production of ligninolytic enzymes was done in an aerated submerged fermentation system with kraft lignin as sole carbon source. The degradation experiment was carried out for 7 days at 30 °C, pH 7. Three potential lignin degraders identified as *Bacillus* sp., *Lysinibacillus* sp. and *Acinetobacter* sp. were successfully isolated. The bacterial growth and secretion of extracellular ligninolytic enzymes confirmed metabolism of kraft lignin by the identified strains. *Lysinibacillus* sp., a novel lignin degrader showed highest manganese peroxidase (76.36 ± 15.74 U/L) and laccase activity (70.67 ± 16.82 U/L) after 7 and 6 days of incubation respectively, while maximal activity of lignin peroxidase (262.49 ± 0.92 U/L) was recorded after 7 days in culture supernatants of *Bacillus* sp. With respect to the activity of the secreted enzymes, the lignin degrading potential of these bacterial strains can be explored in the valorisations of lignocellulosic biomass in industrial processes such as pulping, bioethanol production, fine chemicals and materials synthesis.**

***Keywords* – Enzymes; kraft lignin; laccase; peroxidases; termites**

1. INTRODUCTION

Termites thrive in great abundance in the terrestrial ecosystems and play a crucial role in depolymerization and mineralization of complex biopolymers in the tropics and subtropics [1], [2]. This is achieved by their highly specialized hind gut flora which produces acetate and other volatile fatty acids from the polysaccharide components and plant lignocelluloses complexes [1].

Lignin is an aromatic polymer of phenylpropanoid units which is found closely bound to hemicellulose and cellulose in the plant cell wall. It plays an important role in the transport of water and protection from pathogens [3], [4]. Lignin structural complexity, as well as its close association and chemical cross-linking with the carbohydrate fraction of plant cell wall makes enzymatic degradation difficult. Lignin biodegradation is a key step in the recycling of carbon in the ecosystem, and in the release of more easily degradable polysaccharides which can be utilized by other micro-organisms incapable of attacking the lignin component of wood [5]. Recently, there are significant interests in the biodegradation of lignin [6], as its

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presence impedes the maximum utilization of cellulose [7], [8] either for pulp or bioethanol production [9]–[11].

A controlled biocatalytic breakdown of lignin is also a potentially valuable source of renewable aromatic and phenolic bio-products, which would be valuable raw materials for the food and flavour industry, and for fine chemicals and materials synthesis [12]. Activity of microorganisms in lignin metabolism is one of the tenable evolutionary inceptions for the degradation of aromatic xenobiotics and environmental pollutants such as polychlorinated biphenyl.

Considerable efforts have been focused on ligninase enzymes production by white-rot and brown-rot fungi which are able to mineralize lignin. These fungi secrete a range of extracellular enzymes capable of degrading lignin which includes heme-dependent lignin peroxidases, manganese peroxidases, versatile peroxidases, and copper-dependent laccases [13]–[16]. However, long weeks of incubation and ligninase enzymes expression in fungi being a secondary metabolism have limited its commercial deployment [17].

Lignin modification by some Actinomycetes [18] and soil bacteria such as *Norcadia*, *Rhodococcus* and *Pseudomonas* sp. have also been reported [18], [19]. Ligninolytic bacteria, however, have not been extensively studied and, thus many ligninolytic enzymes may await discovery. Delignification by bacteria for biotechnological approach may offer several advantages over fungi in terms of large-scale growth efficiency, thermo stability and convenience in molecular genetics and protein expression [17], [20].

Coptotermes curvignathus is a common pest in Malaysia, notorious for its attack on rubber and oil palm plantations. The role of the gut flora of this termite in the degradation of cellulose has been investigated. However, no study has been carried out on the production of ligninase enzymes from the gut symbionts of *Coptotermes curvignathus.* This research, therefore, aims to screen and identify ligninolytic enzymes producing bacteria from the gut of *Coptotermes curvignathus*. The enzyme production was done in submerged fermentation system using kraft lignin as substrate.

2. MATERIALS AND METHODS

2.1. Samples Collection and Preparation

Bacteria isolates from termites' gut were obtained from the Department of Crop Science, University Putra Malaysia, Sarawak. The isolates were re-streaked on LB agar plates for 24 hrs at 37 °C and stored in 25 % glycerol stock, prepared by mixing equal volumes of 50 % glycerol and overnight cultures of the isolates, at –70 °C.

2.2. Screening of Lignin Degrading Bacteria

The isolates were assayed for their lignin degrading capacity based on their ability to grow on Mineral salt media (MSM) supplemented with kraft lignin as sole carbon source, as well as their ability to decolorize commercialized dyes.

2.2.1. Growth on MSM-Lignin Agar

A selection protocol based on the ability to utilize kraft lignin as sole carbon source was used to select bacteria isolates capable of degrading lignin. MSM-Lignin agar was prepared according to Bandounas et al. [21] 200 mL M9 Salt solution (64 g $Na_2HPO_4.7H_2O$, 15 g $KHPO_4.7H_20$, 2.5 g NaCl, 5.0 g NH_4Cl, 1 L ddH_2O), 100 µL 1M CaCl, 2 mL 1 M $MgSO_4$, 5 g of lignin powder, 15 g of agar powder, 800 ml of ddH_2O and autoclaved at 120 ºC for

20 minutes. Formation of colonies was observed on MSM-Lignin agar plates incubated for 7 days at 37 °C.

2.2.2. Dye Decolourization Assay of Isolates

Decolorization of lignin-mimicking dyes has been reported to be evident of production of ligninolytic enzymes [19]. The isolates which showed good growth on MSM-Lignin agar were further screened for their ability to express ligninase enzymes using indicator dyes such as methylene blue and azure B as described by Bandounas et al. [21], colonies on MSM-Lignin agar were streaked on LB agar plates supplemented with 2 g/l and 2 g/l methylene blue and azure B respectively. The plates were incubated at 37 °C for 72 hrs and observed for zone of clearance.

2.3. Identification of Ligninolytic Enzyme-Producing Microorganisms

The Microbial genomic DNA was extracted from the pure culture of selected bacterial isolates using DNeasy Qiagen Kit according to manufacturer's instruction. The 16S rRNA gene amplification and sequencing were carried out by First Base Laboratory, Malaysia. The PCR products were analysed on 0.8 % (w/v) agarose gel and sequenced. All sequences were blasted using BLAST against the NCBI 16S ribosomal RNA sequences (Bacteria only) Database, excluding uncultured Bacteria bacterium (taxid: 77133). Sequence data were aligned with software package MEGA and the Phylogenetic tree was constructed using the neighbour-joining method.

2.4. Enzyme Production in Submerged Fermentation

Production of ligninase enzymes was done via a continuous submerged fermentation system [22]. Kraft lignin degradation experiments were conducted under aerobic conditions at 30 °C pH 7 on a rotary shaker (120 rpm) in LB medium supplemented with lignin as sole carbon source. The crude enzyme was extracted by centrifugation. The supernatant containing the crude enzyme extract was used to assay for enzymes activity, mentioned in 2.6.

2.5. Bacterial Growth

The growth rates of the isolates were determined by measuring the OD_{600} of cultured samples withdrawn at intervals. Centrifuged uninoculated medium was used as a control. The control and cultured samples were centrifuged at 5000 rpm for 20 min to remove biomass and suspended solids. The supernatant containing the crude enzyme extract was used to assay for enzymes activity, mentioned in 2.6.

2.6. Enzymes Assay

Lignin peroxidase (LiP) activity was measured by monitoring the oxidation of veratryl alcohol to veratryl aldehyde in the presence of H_2O_2, this was determined by the increase in absorbance at 310 nm. ($\varepsilon 310 = 9300\ M^{-1}\ cm^{-1}$) in 100 mM citrate buffer (pH 3.0) at 30 °C. Meanwhile, manganese peroxidase (MnP) activity was assayed spectrophotometrically with phenol red ($\varepsilon 610 = 22000\ M^{-1}\ cm^{-1}$) as substrate in 50 mM sodium lactate at (pH 5.0) 30 °C [23]. Finally, laccase (Lac) activity was measured using 2,2-azinobis (3-ethylbenzthiazoline-6- sulphonic acid) (ABTS) in 0.1M acetate buffer (pH4.5) at 30 °C. Oxidation of ABTS was determined by the increase in A420 ($\varepsilon 420 = 36\ 000\ M^{-1}\ cm^{-1}$) [24]. The enzyme activity was expressed in international units (U), defined as the amount of enzyme required to convert 1 μmole of substrate to product per minute.

3. STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was carried out for the analysis. The results obtained and significant differences ($p < 0.05$) were determined using Minitab version 17.0.

4. RESULTS AND DISCUSSION

4.1. Screening of Lignin Degrading Microorganisms

Aliquots of enrichment cultures were plated on MSM-lignin agar and further streaked on LB medium supplemented with lignin-mimicking dyes. Ten isolates out of 40 showed positive results for lignin degradation based on the qualitative screening on the selective agar and indicator dyes. This was evident based on the good growth observed on agar plates supplemented with kraft lignin as carbon source, as well as the formation of zone of clearance on methylene blue and azure b plates. The isolated were designated TG003, TG007, TG CH4, TG015, TG 016, TG 017, TG CH2, TG084, TG108, and TG101.

4.2. Identification of Ligninolytic Microorganisms

The ten selected isolates were identified from 16s RNA sequences. The phylogenetic analysis of the selected bacterial strains revealed that TG003, TG007, TG CH4, TG015, TG016, TG 017, TG CH2 formed a clade with Bacillus genus. TG084 was also shown to exhibit maximum homology with *Lysinbacillu*s genus. Isolate TG 101 had previously been identified as *Acinetobacter iwoffi*. The isolates were therefore designated as *Bacillus* sp., *Lysinbacillus* sp. and *Acinetobacter iwoffi* respectively according to 16S rRNA gene identification.

The termite gut provides excellent habitat for lignocellulolytic microorganisms. These organisms produce acetate and other volatile fatty acids from the polysaccharide components and plant lignocelluloses complexes [1].

There are several reports on isolation of *Bacillus species* from termites' gut. *Bacillus* spp. have been identified in the gut of *Zootermopsis angusticollis* [25], [26], *Odontermes* sp. and *Macrotermes* sp. [27]. Cellulolytic *Bacillus cereus* has also been reported from the gut of *Coptotermes curvignathus* [28]. However, few reports on the lignin degradation capabilities of *Bacillus species* have been reported. Bandounas [21] reported the capability of *Bacillus* spp. to grow on kraft lignin and ligninolytic indicator dyes, with particular preference for recalcitrant phenothiazine dye class (Azure B, Methylene Blue and Toluidene Blue O). However, growth on kraft lignin was dependent on the incorporation of copper sulphate and yeast extract into the growth medium. This study reports the degradation of kraft lignin without the addition of co-substrates. Raj et al. [29] reported the ability of *Bacillus* spp. to decolourise kraft lignin after 6 days of incubation. This is consistent with the findings of this study.

There are no reports on the identification of *Lysinbacillus* spp. from termite's gut nor has the ligninolytic potential of this bacterial strains being reported. However, *Lysinbacillus* spp. were found to be present in dye contaminated soil [30], [31]. There are also reports of strains of *Lysinbacillus* sp. isolated from dye contaminated soil sample that are able to decolorize sulfonated azo dyes [32], [33] and industrial effluents [30]. This lends credence to the suggestion of a plausible link between aromatic degradation and lignin degradation [34], which is rational given that much of the aromatic material present in the soil are derivatives of lignin.

Acinetobacter sp. has been isolated from the gut of wood and bark inhabiting long horned beetles [35], and also in the gut of *Zootermopsis nevadensis* [25]. *Acinetobacter* sp. has been also identified in a consortium reported to degrade 60.9 % lignin in reeds within 15 days of cultivation. *Acinetobacter* sp. is clustered into the family of Gammaproteobacteria which is consistent with the classes of bacteria reported to be major lignin degraders; Alphaproteobacteria, Gammaproteobacteria and Actinobacteria [17]. Noteworthy, this observation suggests the existence of metabolic capabilities in these groups that aid in degradation of lignin. Degradation of biphenyl component of lignin has been reported in *Acinetobacter* and *Bacillus* genera [36]. Metabolism of this component is crucial in lignin degradation.

4.3. Bacterial Growth

In order to determine the lignin degrading capacity of the identified isolates, cultures of each strain were grown in LB medium supplemented with kraft lignin. Whilst, all strains showed good growth in kraft lignin without the addition of co-substrates, some strain grew much more swiftly than others (Fig. 1). This is an indication of the differences in the metabolism rate of kraft lignin exhibited by the isolates. *Lysinbacillus* sp. grew much more rapidly reaching optical density of 0.3 after 24 hrs and 1.5 after 144 hrs, compared to *Acinetobacter* sp. (0.2 and 1.4) and *Bacillus* sp. (0.1 and 1.0) respectively. In contrast to the continual increase in cell density observed in culture of *Acinetobacter* sp. over the 7-day cultivation period, *Lynsibacillus* sp. and *Bacillus* sp. slowly decreased from a peak of 1.5 and 1.4 on day 6 to 1.4 and 1.3 respectively after 7 days of incubation. The decline in the cell density suggests the release of low weight molecular compounds which exhibit inhibitory effects on the growth of the bacterial strains.

4.4. Enzymes Assay

Extracellular peroxidases (lignin peroxidase, manganese peroxidase) and laccases which are the main ligninolytic enzymes have been characterized in microorganisms [4]. These enzymes show variations in characteristics with the microbial sources. The ability of an organism to produce either one or more of these enzymes also differs among microbes [37]. In this study, we investigated the ability of selected bacterial isolates in the production of LiP, MnP and laccase. Interestingly, activities of all three enzymes were detected in culture supernatants of all bacterial strains. This is consistent with a previous report on the biodegradation of kraft lignin by *Comamonas* sp. B-9 isolated from eroded bamboo slips [25]. However, ligninolytic enzyme systems reported in *Pandoraea* sp. B-6 isolated from bamboo slips lacked lignin peroxidase [38].

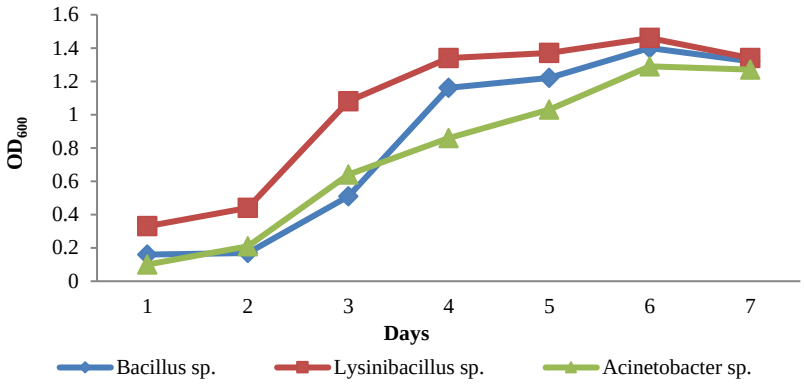
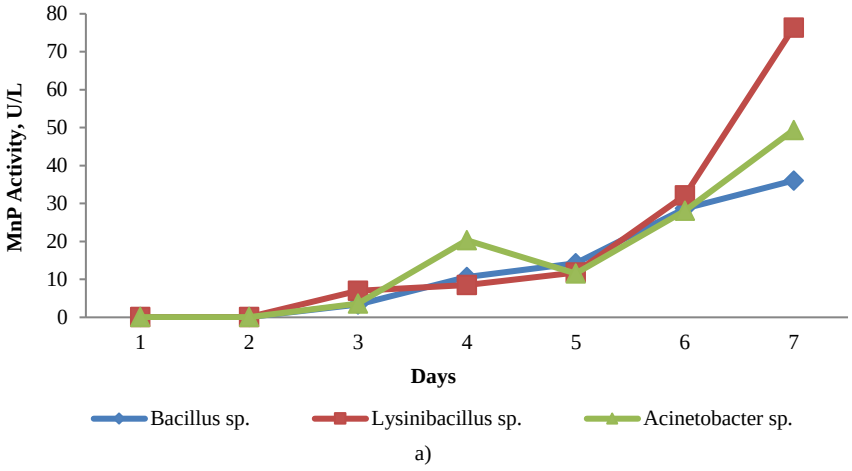


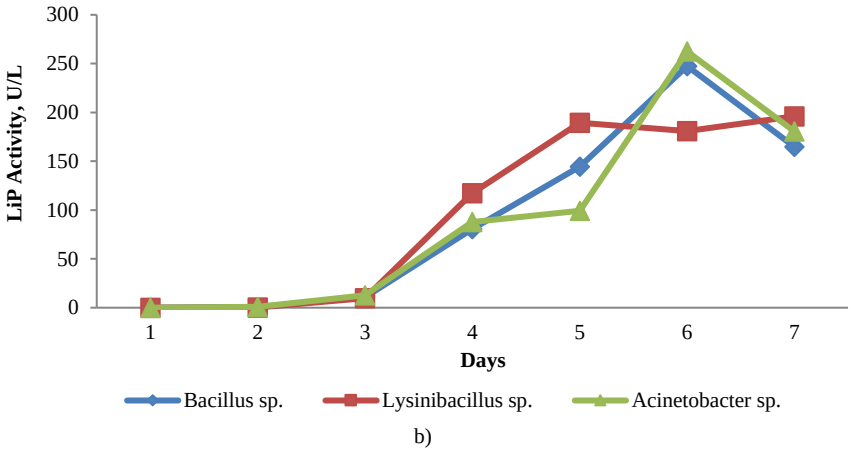
Fig. 1. Cell density (OD_{600}) of cultures of lignin-degrading gut isolates of *Coptotermes curvignathus* grown in LB medium supplemented with kraft lignin.

Table 1 show that maximum production of ligninolytic enzymes was reached on the 6th (LiP and Laccase) and 7th day (MnP) of incubation. Peak LiP activity was reached on the 6th day of incubation during the production of ligninolytic enzymes from oil palm plantation soils [39]. This is consistent with the findings of this study. In contrast to MnP, LiP and laccase activities declined toward the 7th day of incubation. This could be explained by the accumulation of low molecular weight lignin fragments which exhibit inhibitory effects on enzyme productions [39]. However, low levels of MnP were detected in the initial 48 hrs of incubation; it saw a significant increase after 72 hrs, and continually increasing through the following hours, reaching maximal after seven days of incubation (Fig. 2). It could be that the released low weight molecular compounds stimulated MnP production rather than inhibit as observed for LiP and laccase. Although, this is at variance with a previous study, where MnP activity was reported to significantly increase in the initial three days of incubation and declined through to the 7th day of incubation. The disparity could be attributed to the variations in microbial sources and production parameters employed in both studies. The activity of ligninolytic enzymes is largely dependent on temperature, pH and presence of inducers [40].

Maximum LiP activity was recorded after 6 days of fermentation for *Bacillus* sp. (247.52 ± 4.8 U/L) and (262.49 ± 0.92 U/L) for *Acinetacbacter* sp. However, *Lysinibacillus* sp. reached peak activity (196.07 ± 1.43 U/L) after 7 days of incubation. MnP peroxidase activity was the highest in culture supernatants of *Lysinibacillus* sp. (76.36 ± 15.74 U/L), compared to that observed in *Acinetobacter* sp. (49.39 ± 5.50 U/L) and *Bacillus* sp. (36.06 ± 1.462 U/L) after 7 days. Optimal laccase activity was reached after 6 days with the highest activity recorded in *Lysinibacillus* sp. (70.67 ± 16.82 U/L) and (50.74 ± 6.41 U/L) for *Acinetobacter* sp., respectively. In comparison to all species, *Bacillus sp* showed the least laccase activity (46.48 ± 27.4 U/L).



a)



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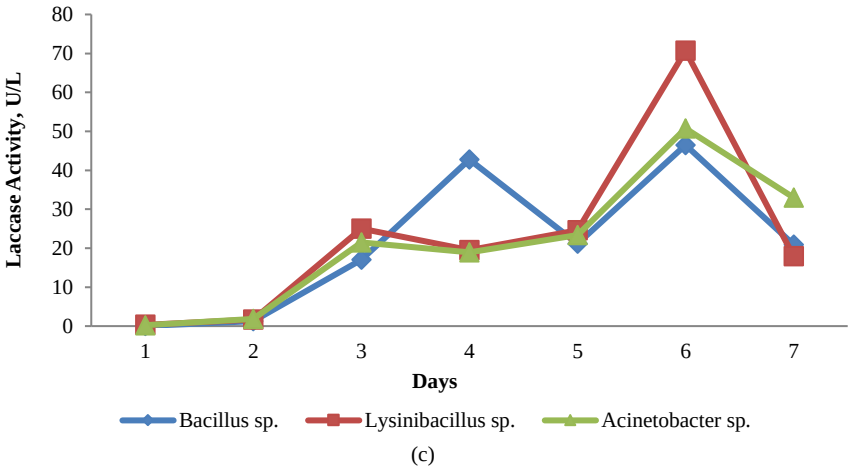

(c)

Fig. 2. Enzyme activities of a) MnP; b) LiP; c) Laccase during degradation of kraft lignin in submerged fermentation.

TABLE 1. OPTIMAL ACTIVITY OF LIGNINOLYTIC ENZYMES SECRETED BY THE ISOLATES

Isolates	Lignin peroxidase		Manganese peroxidase		Laccase	
	Enzyme activity, U/L	Day	Enzyme activity, U/L	Day	Enzyme activity, U/L	Day
Bacillus sp.	247.52 ± 4.8	6	36.06 ± 1.462	7	46.48 ± 27.4	5
Lysinibacillus sp.	196.07 ± 1.43	7	76.36 ± 15.74*	7	70.67 ± 16.82*	5
Acinetobacter sp.	262.49 ± 0.92*	6	49.39 ± 5.50	7	50.74 ± 6.41	5

*The highest species' enzyme activity.

Results indicate that activities of LiP (262.49 ± 0.92 U/L) was higher than that of MnP (76.36 ± 15.74 U/L), as well as Laccase (70.67 ± 16.82 U/L) in all isolates. This suggests that LiP could be the predominating enzyme in the ligninolytic enzyme systems of gut flora of *Coptotermes curvignathus*.

5. CONCLUSIONS

This study reports the lignin degrading capability of gut flora of *Coptotermes curvignathus*. Three bacteria isolates; two belonging to classes of previously known bacterial lignin degraders (*Bacillus* sp. and *Acinetobacter* sp.) and a novel lignin degrader (*Lysinibacillus* sp.) were identified. All bacterial strains were able to metabolise kraft lignin. However, *Lysinibacillus* sp. grew much more rapidly than others. Activities of all three enzymes were detected in culture supernatants of all bacterial strains studied. *Lysinibacillus* sp. showed optimal MnP and laccase activity, while highest LiP activity was observed in culture supernatant of *Acinetobacter* sp. The ability of the bacterial strains to produce LiP, MnP and laccase is advantageous for efficient delignification and can be explored for potential biotechnological applications in areas of pulping, bioremediation and bio-ethanol production. Not much is known about the novel *Lysinibacillus* sp. and further studies is required in order to explore the ligninolytic potential of this bacterium industrially. Optimization of the production parameters and purification of the secreted enzymes is also required to enabling better characterization of these enzymes and enhancing their activity.

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