Production of Ligninolytic Enzymes by Newly Isolated Bacteria from Palm Oil Plantation Soils

Nor Hashimah Abdul Rahman, Nor'Aini Abdul Rahman,* Suraini Abd Aziz, and Mohd Ali Hassan

Three aerobic lignin-degrading bacterial strains were isolated from palm oil plantation soils. The bacterial isolates were screened using a selective nutrient medium of minimum salt media (MSM), with kraft lignin as lignin substrate and methylene blue as the ligninolytic dye indicator. The newly isolated bacterial strains SHC1, SHC2, and SHC3 were found to have the potential to tolerate high concentrations of kraft lignin and produced all three main ligninolytic enzymes (lignin peroxidase, manganese peroxidase, and laccase); these strains may therefore be useful in the degradation of lignin in oil palm empty fruit bunch biomass. The production of ligninolytic enzymes was carried out by means of submerged fermentation for 7 days using 2 mm of oil palm empty fruit bunch (OPEFB) fiber as a substrate. These bacterial isolates were characterized using biochemical tests from Biolog and identified using 16S rRNA gene sequencing analysis, which identified the strains SHC1, SHC2, and SHC3 as *Bacillus* sp., *Ochrobactrum* sp., and *Leucobacter* sp., respectively with 99% sequence similarity. *Bacillus* sp. SHC1 produced the highest manganese peroxidase (MnP) of 2313.4 U/L on the third day and the highest lignin peroxidase (LiP) of 209.30 U/L on the fifth day of fermentation. The optimum pH and temperature for the production of ligninolytic enzymes by *Bacillus* sp. SHC1 were pH 8 and 30 °C.

Keywords: Lignin degrading bacteria; Ligninolytic enzymes; Palm oil plantation soils; Oil palm empty fruit bunch (OPEFB)

Contact information: Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia;
**Corresponding author: nor_aini@upm.edu.my*

INTRODUCTION

The palm oil industry in Malaysia generates a large amount of biomass, up to 80 million dry tones in 2010. Judging from the current rate of increasing palm oil plantation yield in 2020, the palm oil sector is expected to generate about 100 million dry tonnes of solid biomass, which includes oil palm empty fruit bunches (OPEFB), palm kernel shells (PKS), mesocarp fibres (MF), oil palm fronds, and trunks (Malaysia Innovation Agency 2011). In spite of extensive studies worldwide over the past several decades investigating the potential exploitation of biomass for biofuel production, there has been no successful large-scale commercialization of the process because of industrial and economical barriers. Therefore, the National Biomass Strategy 2020 has been established to utilize biomass, especially lignocellulosic biomass to produce higher value-added products such as biofuel and other industrial applications in Malaysia.

Fundamentally, biomass consists of cellulose, hemicelluloses, and lignin (Sun and Cheng 2002). Among them, lignin is the most structurally complex, possessing a high molecular weight and the most recalcitrant consisting of various biologically stable

linkages (Pèrez *et al.* 2001). The disruption of lignin is crucial for enzymatic hydrolysis to occur, as lignin acts as a barrier to the degradation of biomass. Chemical approaches such as acid hydrolysis, aqueous ammonia pre-treatment, and steam explosion are some of the existing pre-treatment techniques for the removal of lignin (Brodeur *et al.* 2011). However, these methods require high cost and extensive energy and they also generate toxic by-products (Harmsen *et al.* 2010). For this reason, improved biological methods including those involving the use of fungi, actinobacteria, bacteria, and enzymes appear to be more cost-effective and more environmental friendly. Most of the research on the microbial degradation of lignin is focused on white-rot and brown-rot fungi because they are the most efficient lignin degraders in nature (Eriksson *et al.* 1990; Leonowicz *et al.* 2001. However, commercialization of lignin degradation by fungi has disadvantages in the form of problems related to fungal protein expression and genetic manipulations (Chandra *et al.* 2008). In addition, fungi showed a lack of stability under practical treatment conditions involving high pH, oxygen limitation, and high lignin concentrations (Raj *et al.* 2007). For this reason, studies on the bacterial degradation of lignin via the production of ligninolytic enzymes have seen increasing research interest. Previous studies showed that bacterial strains can degrade lignin and lignin-related model compounds (Bugg *et al.* 2011).

The objective of this research was to discover lignin-degrading bacteria present in palm oil plantation soils that produce all three main ligninolytic enzymes for degradation of lignin in oil palm empty fruit bunch (OPEFB) biomass. In this study, isolation and identification of bacterial strains with a high tolerance for kraft lignin was achieved. The isolated lignin-degrading bacteria were screened for their ability to grow on minimal salt medium (MSM) agar containing kraft lignin as the sole carbon source. Decolorization of methylene blue dye was used as an indicator of the oxidation ability of ligninolytic enzymes produced by the potential lignin-degrading bacterial strains. Three bacterial isolates were chosen for further study in submerged fermentation to evaluate the production of the three main lignin-degrading enzymes; lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac). The effects of pH and temperature were also tested to improve the production of extracellular ligninolytic enzymes in submerged fermentation using OPEFB fibers as substrate.

EXPERIMENTAL

Materials

The chemicals and reagents used in this study were analytical grade. Kraft lignin, 2,2'-azinobis-(3-ethylbenzethiazoline-6-sulphonate) (ABTS), guaiacol, monopotassium phosphate, dipotassium phosphate, ammonium nitrate, sodium acetate, veratryl alcohol and methylene blue were purchased from Sigma-Aldrich (USA). Magnesium sulfate and calcium chloride were obtained from Merck (USA). Luria-Bertani (LB) broth, Miller was acquired from BD (Becton, Dickinson and Company, USA). All solutions were prepared in deionized water. Kraft lignin used in this study is a polymeric lignin alkali material from the pulp and paper industry resulting from alkaline sulfide treatment of lignocelluloses which has been widely used for lignin-related studies (Shi *et al.* 2013). Oil palm empty fruit bunch (OPEFB) was obtained from Sri Ulu Langat Palm Oil Mill in Dengkil, Selangor, Malaysia. The shredded OPEFB was soaked in detergent to remove

any residual oil and then dried in an oven at 50 °C overnight. The OPEFB fibers were ground by a grinder to an average size of 2 mm.

Methodology

Site and sample collection

Soil samples were collected from three different sampling sites in Felda Serting Hilir (2° 59' 31.30" N, 102° 28' 54.62" E), Negeri Sembilan, Malaysia. The samples were collected from decayed palm oil biomass sites, pruned frond sites, and an oil palm empty fruit bunch (OPEFB) mulching sites. The soils were collected from the surface to a depth of about 0 to 10 cm using sterile spatulas and transported to the laboratory and stored at -20 °C. For molecular biological analysis, sub-samples were stored at -80 °C.

Soil characteristics

Soil characteristics were determined using standard methods (APHA 1985). For each sampling site, three soil samples were combined and mixed together to construct a composite sample. Acid detergent fiber (ADF), neutral detergent fiber (NDF), and acid detergent lignin (ADL) methods were used to determine the cellulose, hemicellulose, and lignin compositions, respectively, of the samples (Goering and Van Soest 1970).

Bacterial isolation and screening

For isolation of the soil bacteria, 1 g of soil sample was added to 100 mL of sterile 0.9% NaCl. The solution was stirred vigorously and then allowed to settle. One milliliter of the liquid mixture was serially diluted until a dilution of 10^6. Then, 100 µL of this solution was plated on minimal salt media containing kraft lignin (MSM-KL) as sole carbon source. MSM-KL media consisted of (g/L of deionized water) KL, 0.5; K_2HPO_4, 4.55; KH_2PO_4, 0.53; $CaCI_2$, 0.5; $MgSO_4$, 0.5; NH_4NO_3, 5 (modified from Chandra *et al.* 2008). The plates were incubated at 30 °C for 7 days until colonies developed. The isolated bacteria were plated onto fresh MSM-KL agar plates repeatedly to obtain pure cultures. Forty-four isolates were obtained and grown in Luria-Bertani (LB), Miller broth medium. Then, 2 mL aliquots of these cultures were frozen as stock cultures in 80% glycerol stock and maintained at -80 °C.

The bacterial isolates were further screened using methylene blue dye as an indicator of the oxidation process by ligninolytic enzymes (Bandounas *et al*. 2011). The isolated bacteria were streaked onto dye-containing agar plates containing MSM agar and 0.5 g/L of kraft lignin as lignin substrate. Methylene blue dye was added at 0.25 g/L and the plates were incubated at 30 °C for 7 days. The agar plates were monitored daily for bacterial growth and decolorization of the methylene blue. Six potential isolates were selected from the large clear (decolorized) zones of methylene blue dyes. Then, these isolates were grown on MSM-KL agar media with different concentrations of kraft lignin (0.5 g/L, 0.6 g/L, 0.7 g/L, 0.8 g/L, 0.9 g/L, 1.0 g/L, and 1.1 g/L) at 30 °C for 7 days. Only three bacterial strains showing rapid growth on MSM-KL agar with 900 mg/L of kraft lignin and were selected for further study. The isolates were purified by at least five successive passages onto fresh MSM-KL. The purity of each strain was ensured by microscopic observation of colony shape, texture and pigmentation.

16S rRNA gene sequence analysis

The complete genomic DNA of each strain was extracted from the pure cultures using a PowerSoil® DNA Extraction Kit (MO-BIO, USA). The DNA extraction was

performed according to the manufacturer's instruction. The partial 16S rRNA gene sequences were amplified via polymerase chain reaction (PCR) using universal eubacterial primers of 27F (5'- AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'- GGT TAC CTT GTT ACG ACT T-3'). One microliter of DNA template was added to 50 µL of reaction mixture containing 5 µL of 10X buffer, 4 µL of dNTP, and 0.5 µL of each primer with concentrations of 20 pmol μL^{-1}, 0.2 µL of exTaq DNA polymerase (TAKARA BIO INC, Japan), and 38.8 µL of sterilized ultrapure water. Thirty cycles of the PCR were performed. Each cycle consisting of denaturation at 96 °C for 5 min, primer annealing at 50 °C for 5 min, and primer extension at 72 °C for 1.5 min. Approximately 1.5 bp of amplified PCR products were purified using an Agencourt AMPure XP (Beckam Coulter, USA) according to the manufacturer's instruction. This quantity was sent for sequence analysis. The purified PCR products of the 16S rRNA genes were sequenced using an Applied Biosystems 3730xl DNA Analyzer (USA). The resulting sequences were analyzed using the biological sequence alignment editor (Bioedit) (Hall 2007). The partial sequences were submitted to basic local alignment search tool (BLAST) (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*) analysis using the online option database at the National Center for Biotechnology Information (NCBI) to determine the identity of the bacterial isolates. The sequences were aligned using the ClustralW program, and a phylogenetic tree was constructed based on a neighbor-joining algorithm using MEGA 5.1 software (Tamura *et. al*. 2011).

Biochemical identification tests

The isolated bacterial strains were identified and characterized by morphology and various biochemical tests using the BIOLOG kit (BIOLOG Inc., USA). Single colonies of bacterial strains were prepared according to the manufacturer's instruction.

Submerged fermentation

One loopful of each selected bacterial strain (SHC1, SHC2, and SHC3) was inoculated into 10 mL of LB medium at an initial concentration of 10^7 cells/mL. Samples were incubated at 30 °C with shaking at 120 rpm for 10 h to obtain a final OD_{600} of 1.0. Then, the inoculums were transferred to 250 mL Erlenmeyer flask containing 90 mL of LB medium and 0.9 g/L of OPEFB fibers. The cultures were incubated at 120 rpm and at 30 °C for 7 days. The initial pH was set at 7.6. Sampling was performed every 24 h to monitor daily bacterial growth (OD). *Escherichia coli* JM109 strain (PROMEGA, USA) was used for negative control.

Preparation of crude enzymes

Samples were collected every 24 h, and their turbidity was measured by reading the OD_{600}. The supernatants and pellets were separated by centrifugation at 8,000 rpm for 10 min. The supernatants were collected and were used to measure extracellular ligninolytic enzyme activity.

Lignin peroxidase enzyme assay

The lignin peroxidase (LiP) activity was assayed via the oxidation of veratryl alcohol to veratryaldehyde at 310 nm (Tien and Kirk 1988). Two milliliters of enzymatic assay consisted of 0.4 mL of citrate-phosphate buffer (100 mM, pH 2.7), 0.1 mL of veratryl alcohol (20 mM), and 0.5 mL of fluid sample. Forty microliters of H_2O_2 (20 mM), which was freshly prepared daily, was added to start the reaction.

The conversion to veratraldehyde was monitored in 1 mL quartz cuvette at 310 nm wavelength using UV-Vis spectrophotometer. One unit of enzyme activity corresponded to the oxidation of 1 micromole veratraldehyde converted from veratryl alcohol per minute under the assay conditions with a molar extinction coefficient of $\epsilon 310 = 9300\ M^{-1}\ cm^{-1}$.

Manganese peroxidase enzyme assay

Manganese peroxidase (MnP) activity was measured via the oxidation of guaiacol to a colored product using the UV-Vis spectrophotometer at 465 nm (Li *et al.* 2008). The enzymatic assay of 1.0 mL consisted of 0.4 mL of sodium lactate buffer (100 mM, pH 4.5), 0.1 mL of guaiacol (1 mM), 0.1 mL of $MnSO_4$ (1 mM), and 0.4 mL of the supernatant of the sample.

The reaction was started by the addition of 30 µL of H_2O_2 (0.1 mM), which was freshly prepared daily. The formation of the colored product was measured in 1 mL quartz cuvette at 465 nm wavelength. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 micromole of colored product per minute at 30 °C under the assay conditions with a molar extinction coefficient of $\epsilon 465 = 12100\ M^{-1}\ cm^{-1}$.

Laccase enzyme assay

Laccase activity was determined by oxidation of 2,2'-azinobis-(3-ethylbenzethiazoline-6-sulphonate) (ABTS) using the method of Wolfenden and Willson (1982). An enzymatic mixture consisted 0.15 mL of 0.03% ABTS, 0.5 mL of 0.1 M sodium acetate buffer at pH 5.0 and 0.35 mL extracellular enzyme samples. The formation of oxidized ABTS was measured in 1mL quartz cuvette (1 cm light path) at 30°C and 530 nm wavelength using UV-Vis spectrophotometer. Oxidation of ABTS was followed by absorbance increase at 420 nm with a molar extinction coefficient of $\epsilon 420 = 36\ 000\ M^{-1}\ cm^{-1}$.

Effect of initial pH

The effect of initial pH was evaluated via submerged fermentation of the selected isolates in LB broth medium with the addition of 0.5 g/L OPEFB powder at 5 different initial pHs.

The optimum initial pH for enzyme production was determined by incubating triplicate samples of the selected isolates at pH 7.0, 7.5, 8.0, 8.5, and 9.0, using 1 M HCl or 1 M NaOH to adjust the pH. The experiments were all conducted in an incubator shaker set at 120 rpm and 30 °C for 7 days. Samples were collected every 24 h. The pH of the medium after growth for 7 days ranged from approximately 8.6 to 8.8.

Effect of cultivation temperature

The optimum temperature for ligninolytic enzyme production was investigated by incubating triplicate samples of the selected isolates in LB broth medium with the addition of 0.5 g/L OPEFB powder at temperatures of 30, 35, 40, 45, and 50 °C. The flasks were incubated at 120 rpm on a rotary shaker for 7 days, and samples were collected every 24 h.

RESULTS AND DISCUSSION

Soil Characteristics

The lignocellulosic properties of the soil samples were assessed to gather information on the palm oil plantation research sites (Table 1). The decayed palm oil biomass site sample showed the highest lignin content, at 0.25%, while the pruned frond sample site showed the highest cellulose and hemicelluloses contents, at 61.11% and 10.38%, respectively. The relatively high cellulose and hemicellulose contents at the pruned frond sample site were due to the pilling of pruned fronds in the soil area. The richness of all three soil sites in lignocellulosic materials such as cellulose, hemi-celluloses, and lignin was due to the decomposition of oil palm biomass (Sulaiman *et al.* 2009). The degradation of lignocelluloses in oil palm plantation soils can be mainly attributed to the activity of the microbial community (Khalil *et al.* 2006). The decayed biomass site sample was found to have the highest soil pH, at 6.99, and the highest total organic carbon content (10.18%) as compared to other samples. The increased soil pH was correlated with the higher organic matter content in soil (Lim and Zaharah 2002).

Table 1. Soil Characteristics from Selected Palm Oil Plantation Sites

Soil Site	Cellulose (%)	Hemicellulose (%)	Lignin (%)	pH	Total Organic Carbon (%)
Decayed biomass	29.11	0.17	0.25	6.99	10.18
Pruned frond	61.11	10.38	0.13	5.77	4.64
OPEFB mulching	47.42	0.24	0.21	6.24	5.23

Isolation and Screening of Lignin Degrading Bacteria

To isolate lignin degrading bacteria, kraft lignin (KL) was added directly to the MSM agar as the sole carbon source. Ten percent of glucose and five percent of peptone were added as additional carbon and nitrogen sources that worked as co-substrates to promote lignin-degrading bacterial growth (Shi *et al*. 2012). Forty-four bacterial strains were obtained, and further screening was carried out by plating the isolate strains in a new MSM-KL medium containing methylene blue as the lignin polymeric dye and incubated for 7 days.

The decolorization of methylene blue has been used previously as an indicator of lignin peroxidase enzyme activity (Ferreira-Leitão *et al.* 2007). Changes in color of the medium from blue to clear were observed for six bacterial strains (SHC1 to SHC6). Following this step, the isolates were further screened without adding glucose or peptone to test their tolerances of kraft lignin. Table 2 shows the bacterial isolates with their tolerances at different concentrations of kraft lignin.

Table 2 shows that SHC1, SHC2, and SHC3 were capable of growing on kraft lignin without glucose and peptone up to 1.0 g/L. A previous report also showed that some lignin-degrading bacteria have been able to use lignin as their sole source of carbon without depending on another carbon source for energy (Chen *et al.* 2012). However, Vicuna (1988) stated that bacteria could not grow without glucose and peptone because these substrates are used co-metabolically. Therefore, in submerged fermentation experiments, glucose and peptone were added to the medium as co-substrates to aid bacterial growth for the co-metabolism of lignin.

Table 2. Bacterial Isolates Grown on MSM-KL Plate without Glucose and Peptone for Measurement of their Tolerances at Different Concentrations of Kraft Lignin

	Kraft Lignin Concentration (g/L)						
Isolates	0.5	0.6	0.7	0.8	0.9	1.0	1.1
SHC1	+++	+++	+++	++	+	+	-
SHC2	+++	+++	+++	++	++	+	-
SHC3	+++	+++	+++	++	+	+	-
SHC4	++	+	+	-	-	-	-
SHC5	++	+	-	-	-	-	-
SHC6	+	-	-	-	-	-	-

+++; very fast growth(grow within 24 hours)
++; fast growth (grow within 48 hours)
+; slow growth (grow within 72 hours)
-; no growth (no grow after 1 week)

The isolates SHC1, SHC2, and SHC3 were able to grow very fast on agar plates containing kraft lignin at a concentration of 0.8 g/L with the addition of glucose and peptone (data not shown). Their growth ability slowly decreased at higher concentrations of kraft lignin. These results were in agreement with a previous study (Chen *et al.* 2012) that used a maximum kraft lignin concentration of 0.3% for *Comamonas* sp. isolates due to the tendency of kraft lignin to inhibit the growth of bacteria at higher concentrations.

Characterization of Bacteria

Figure 1 shows the phylogenetic tree that was constructed based on the linkages of the bacterial strains SHC1, SHC2, and SHC3. The 16S rRNA analysis identified the 16S rRNA gene sequences of SHC1, SHC2, and SHC3 as *Bacillus* sp., *Ochrobactrum* sp., and *Leucobacter* sp., respectively, with 99% sequence similarity. The completed 16S rRNA gene sequences were deposited in the GenBank database under Accession No: KC845229 (SHC1), KC845230 (SHC2), and KC845231 (SHC3). A non-related sequence of *Acidobacterium capsulatum* (NR076636) was also included in the tree as an out-group to demonstrate the linkage proximities of the selected samples. The partial 16S rRNA gene sequence of isolate SHC1 showed 99% similarity to a variety of members of the *Bacillus* genus, such as *Bacillus cereus*, *Bacillus anthracis*, and *Bacillus thuringiensis*. The partial 16S rRNA gene sequence of isolate SHC1 showed the highest similarity to *B. cereus* FR1-35 and *B. cereus* DZ-h. Based on previous studies, *Ochrobactrum* sp. was being mentioned as ligninolytic bacteria (Taylor et al. 2012). In their studies, they identified *Ochrobactrum* sp. LMG 20564 and *Ochrobactrum anthropi* strain which show colorimetric spray assay activity without hydrogen peroxide and with presence of hydrogen peroxide. *Leucobacter* sp.is considered a novel ligninolytic bacterium, as there have been no previous reports of lignin-degrading activity by this strain.

Biochemical testing was also conducted using the GEN III OmniLog identification system (BIOLOG Inc, USA). Table 3 shows several selected biochemical compounds from Biolog biochemical test.

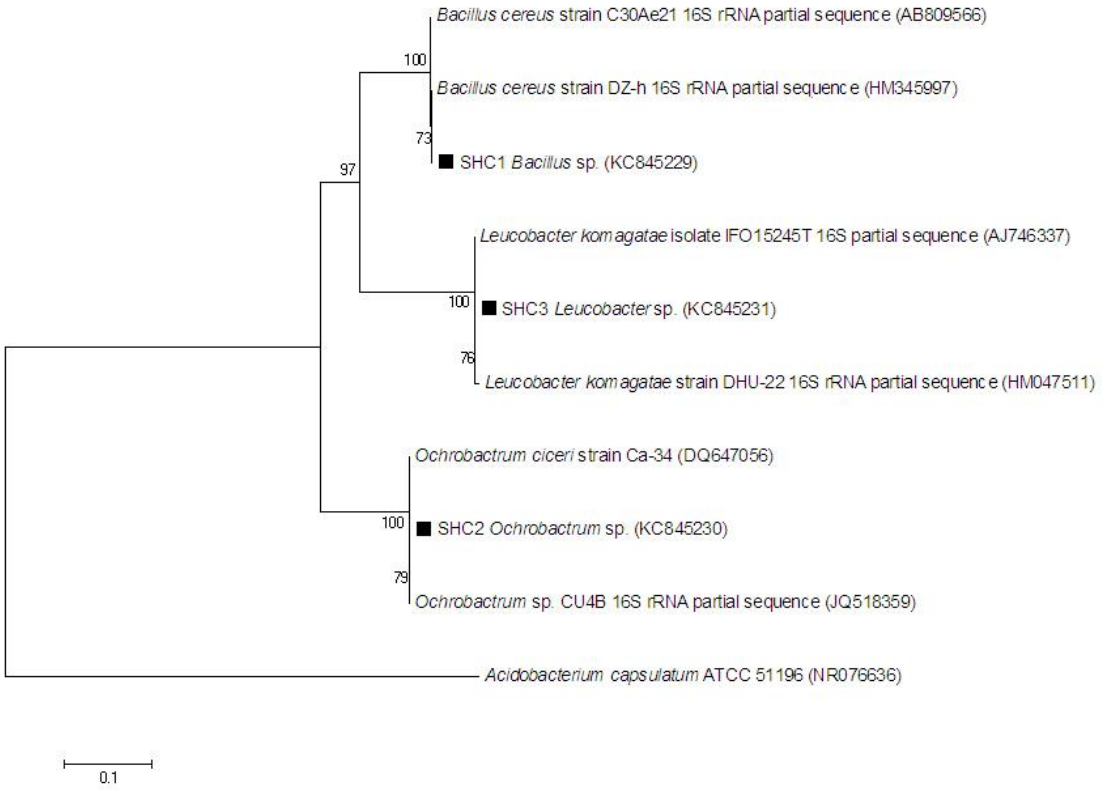

Fig. 1. Phylogenetic tree of partial 16S rRNA sequences based on selected isolated bacterial strains. The tree was constructed using the neighbor-joining method (Saitou and Nei 1987) and the Jukes-Cantor method (Jukes and Cantor 1969) by MEGA5 software (Tamura *et al.* 2011). The scale bar corresponds to a 2% difference, and the bootstrap values are shown at the nodes. The black square symbol represents bacterial isolates, and sequences of type strains from RDP were included for comparison.

Based on the results, isolates SHC1 and SHC2 were judged to be gram-positive bacteria. Isolate SHC3 was gram-negative. All the bacterial isolates were rod-shaped.

Production of Ligninolytic Enzymes

The production of ligninolytic enzymes for these three isolated bacteria was conducted in triplicate experiments to gain better standard results. Strain *Escherichia coli* JM109 was used as a negative control in this experiment. Initially, we used kraft lignin (KL) powder as a substrate in submerged fermentation to measure the production of ligninolytic enzymes from our isolated bacteria. This experiment was conducted to ensure that the ligninolytic enzymes were produced by using specific substrate (in this case, kraft lignin). Unfortunately, the production of ligninolytic enzymes by using KL substrate was lower compared to production of ligninolytic enzymes by using OPEFB substrate (data not shown). The goal here was to produce ligninolytic enzymes using OPEFB waste. Thus, based on submerged fermentation by using OPEFB as substrate, interestingly, all three selected bacterial strains showed positive results toward the three main ligninolytic enzymes (lignin peroxidase, LiP; manganese peroxidase, MnP; and laccase, Lac) in submerged fermentation. Table 4 shows the highest ligninolytic enzymes produced by *Bacillus* sp. SHC1, *Ochrobactrum* sp. SHC2, and *Lactobacter* sp. SHC3 during 7 days of fermentation.

Table 3. Biochemical Compounds of Selected Isolated Bacteria Based on Biolog

Biochemical Compounds	SHC1	SHC2	SHC3
Shape	Rod	Rod	Rod
Gram reaction	Gram positive	Gram negative	Gram positive
d-Cellobiose	+	+	-
Sucrose	+	+	-
d-Turanose	+	+	+
Stachyose	-	-	+
pH 5	-	-	-
pH 6	+	+	+
β-Methyl-*d*-glucoside	-	-	+
N-Acetyl-*d*-glucosamine	+	+	+
1% NaCl	+	+	+
8% NaCl	-	-	-
α-*d*-Glucose	+	+	-
d-Mannose	+	+	-
d-Fructose	+	+	+
d-Galactose	+	+	-
l-Rhamnose	+	+	+
1% Sodium lactate	+	+	+
Glycerol	+	-	-
d-Glucose-6-phosphate	-	+	+
d-Fructose-6-phosphate	+	+	+
Gelatin	-	-	-
l-Alanine	+	+	+
l-Arginine	+	+	+
l-Aspartic acid	+	+	+
l-Glutamic acid	+	+	+
l-Histidine	+	+	+
l-Pyroglutamic acid	+	+	-
Lincomycin	+	+	-
Guanidine hydrochloride	+	+	+
Pectin	+	+	+
d-Galacturonic acid	+	+	-
d-Glucuronic acid	+	+	+
Quinic acid	+	+	+
Tetrazolium blue	+	+	+
p-Hydroxy-Phenylacetic acid	-	-	-
Methyl pyruvate	+	+	+
d-Lactic acid methyl ester	-	+	+
l-Lactic acid	+	+	+
Citric acid	+	+	+
d-Malic acid	+	+	+
Nalidixic acid	-	-	+
Lithium chloride	+	-	+
Tween 40	+	+	-
α-Keto-butyric acid	+	+	+
Acetoacetic acid	+	+	+
Propionic acid	+	+	+
Acetic acid	+	+	+
Formic acid	+	+	+

+; positive, -;negative

Table 4. Highest Lignin Peroxidase, Manganese Peroxidase, and Laccase Enzyme Activities Produced by Selected Bacterial Strains

Isolates	Lignin peroxidase		Manganese peroxidase		Laccase	
	Enzyme activity (U/L)	Day	Enzyme activity (U/L)	Day	Enzyme activity (U/L)	Day
Bacillus sp. SHC1	168.40	6	2009.70	4	2.10	5
Ochrobactrum sp. SCH2	114.20	5	904.30	5	5.67	6
Leucobacter sp. SHC3	153.20	6	1489.20	4	1.90	4

Chandra *et al.* (2008) reported that lignin-degrading bacteria need glucose and peptone as co-substrates to aid in the degradation of lignin. In this study, LB broth containing OPEFB fibers as lignin substrate was used as medium for submerged fermentation. Table 4 shows that the maximum production of ligninolytic enzymes occurred between the fourth and sixth days of incubation time. This report is similar to the previous study in which isolated lignin-degrading bacteria produced the most ligninolytic enzymes during the fourth and fifth days (Ahmad *et al.* 2010). Meanwhile, the production of ligninolytic enzymes declined toward the end of fermentation, between the sixth and seventh days. Based on observations of ligninolytic enzyme assay analysis, the decline in ligninolytic production started between the sixth and tenth days, which varied depending on the bacterium (data not shown). This phenomenon can be explained by the inhibitory effect of some low-molecular weight lignin fragments (Perestelo *et al.* 2008). Figure 2 shows the lowest production rate of the MnP enzyme during the first three days of the incubation. This can be explained by the low growth rate of these strains due to lack of adaptation to the lignin.

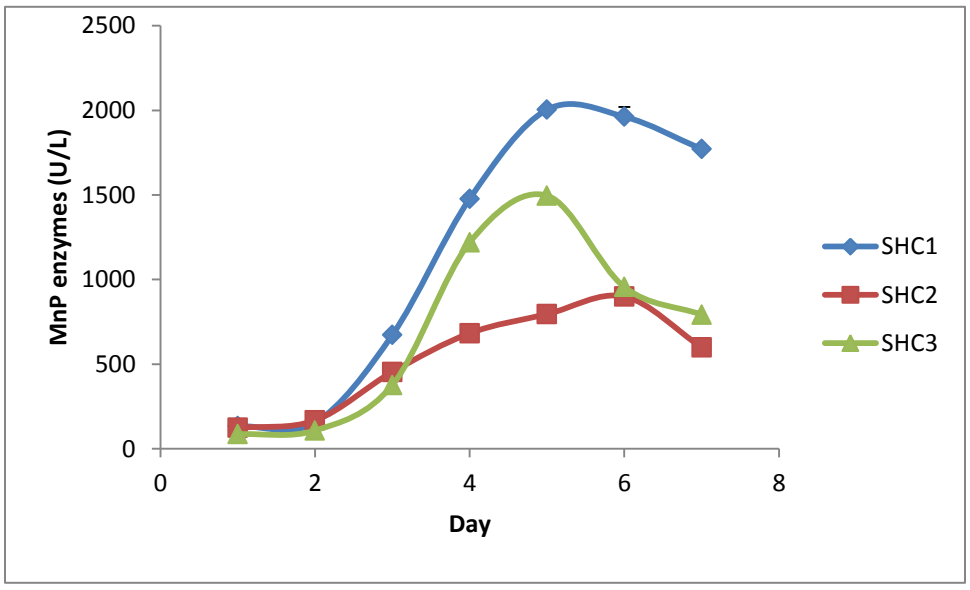

Fig. 2. Profile of manganese peroxidase (MnP) enzyme production by isolates *Bacillus sp.* SHC1, *Ochrobactrum sp.* SHC2, and *Leucobacter sp.* SHC3 in shake flask fermentation

The strain *Bacillus sp. SHC1* showed the highest LiP enzyme activity on the sixth day (168.40 U/L). All three isolated bacterial strains produced the highest LiP enzyme activity on the sixth day and declined toward the seventh day of fermentation. For *Bacillus sp. SHC1* strain, it showed the highest MnP enzyme activity on the fifth day at 2009.70 U/L (Fig. 2). Meanwhile, the strain *Ochrobactrum* sp. SHC2 produced the highest MnP enzyme activity on the sixth day and then dropped drastically after the following days. Meanwhile, the strain *Leucobacter sp.* SHC3 produced the lowest activity of Lac (1.9 U/L). Based on the observation, the Lac enzyme activity is low compared to LiP and MnP in all three strains.

Table 5. Optimum pH for Selected Bacterial Strains and Maximum Ligninolytic Enzyme Activities

Strain	Optimum pH	Lignin peroxidase		Manganese peroxidase		Laccase	
		Enzyme activity (U/L)	Day	Enzyme activity (U/L)	Day	Enzyme activity (U/L)	Day
Bacillus sp. SHC1	8	198.52	5	2214.3	2	2.97	4
Ochrobactrum sp. SCH2	7.5	141.65	5	1008.91	6	6.71	5
Leucobacter sp. SHC3	8	178.44	7	1610.49	4	2.61	4

For effect of pH in the production of lignin degrading enzymes by isolated bacteria, selected pH range from 7.0 to 9.0 were selected according to previous studies of isolated ligninolytic bacteria in Oliveira *et al.* (2009). This report stated that most of ligninolytic bacteria which were isolated from soil positively affected the growth and production of enzymes toward alkaline pH. With respect to the effect of pH on the ligninolytic enzymes, the optimum LiP activity was obtained in the range of pH 7.5 to pH 8. There was a decrease in ligninolytic activity with an increase in pH and complete inactivation shown at pH 9. Shi *et al.* (2013) reported an alkaline pH range for the growth of *Cupriavidus basilensis*, which is a lignin-degrading bacterium. The MnP enzyme activity was optimum at pH 8 for *Bacillus sp.* SHC1. Meanwhile, for Lac enzyme activity, the highest enzyme activity was 6.71 U/L at day 4 for *Ochrobactrum* sp. SHC2.

Table 6. Optimum Temperature with Maximum Ligninolytic Enzyme Activities from Isolated Bacterial Strains

Strain	Optimum temp (ºC)	Lignin peroxidase (LiP)		Manganese peroxidase (MnP)		Laccase	
		Enzyme activity (U/L)	Day	Enzyme activity (U/L)	Day	Enzyme activity (U/L)	Day
Bacillus sp. SHC1	30	209.30	5	2313.4	3	3.00	3
Ochrobactrum sp. SCH2	35	170.55	5	1119.81	6	10.21	5
Leucobacter sp. SHC3	35	197.1	6	1980.10	4	3.89	4

For determination of optimum temperature condition for production of ligninolytic enzymes in isolated bacteria strains, a temperature range from 30 °C to 50 °C was selected due to previous reports by Kuwahara *et al.* (1984) and Zhu *et al.* (2013). It was stated in a previous report that some of the ligninolytic bacteria isolated from soil are thermophilic strains (Oliveira *et al.* 2009).

Based on the results, at 35 °C, 76% of the LiP activity was retained, while further increases in temperature resulted in drastic decreases in activity. This indicated that higher temperatures resulted in gradual inactivation of the LiP (Bibi and Bhatti 2012). For manganese peroxidase, *Bacillus* sp. SHC1 produced the highest enzyme activity at a temperature of 30 °C. For laccase, the bacteria *Ochrobactrum* sp. SHC2 produced a maximum activity of 10.21 U/L on day 5, even though at 35 °C, the LiP and MnP enzyme activities were highest for *Leucobacter sp.* SHC3. The laccase enzyme activity was lower at a temperature of 30 °C.

In previous studies, most of the isolated ligninolytic bacteria could only produce two main ligninolytic enzymes, especially a combination of MnP and Lac enzyme production (Shi *et al.* 2013; Chen *et al.* 2012 and Oliveira *et al.* 2009). Meanwhile, in this study, all three isolated strains produced all three main ligninolytic enzymes: LiP, MnP, and Lac. Regardless of the other findings, only MnP and LiP showed comparable enzyme activity with previous studies, while the Lac enzyme activity results showed the lowest activity. The maximum Lac obtained in this work was from *Ochrobactrum* sp. SHC2 which was 10.21 U/L, while *Comamonas* sp. B-9 is at 1250 U/L based on Chen *et al.* (2012). Shi *et al.* (2013) reported that *Cupriavidus basilensis* B-8 can produce Lac up to 815.6 U/L. Based on the present work, *Bacillus* sp. SHC1 can produce a maximum MnP at 2313.4 U/L, which is higher than for *Cupriavidus basilensis* B-8 at 1685.3 U/L (Shi *et al.* 2013). However, the MnP activity from *Comamonas* sp. is still the highest at 2903.2 U/L, as reported in Chen *et al*. (2012). Based on these results, it can be concluded that the present isolated bacteria can produce ligninolytic enzymes at comparable activity when compared with previous studies.

The results of the present study confirmed that three newly isolated bacterial strains from palm oil plantation soils can grow on kraft lignin as a sole carbon source and can produce three main ligninolytic enzymes. Further studies are required to measure lignin degradation of lignocellulosic biomass by these bacterial strains. Moreover, future studies may determine other optimum conditions in addition to pH and temperature, such as nutrients, dissolved oxygen level, and bacterial concentrations to maximize the production of ligninolytic enzymes by pure or mixed cultures of these newly isolated strains.

CONCLUSIONS

1. Three newly isolated bacterial strains that produced ligninolytic enzymes were isolated from palm oil plantation soils. Isolate SHC1 was identified as *Bacillus* sp. (Accession no.: KC845229), isolate SHC2 was identified as *Ochrobactrum* sp. (Accession no.: KC845230), and isolate SHC3 was identified as *Leucobacter* sp. (Accession no.: KC845231). These strains utilized lignin as the sole source of carbon and produced the main ligninolytic enzymes; lignin peroxidase, manganese peroxidase, and laccase.

2. Based on the results obtained in this study, the isolate *Bacillus* sp. SHC1 was chosen as the best of the ligninolytic bacterial strains, as it achieved maximum manganese peroxidase production of 2313.4 U/L on the third day under the optimum conditions of pH 8 and 30 °C. Strain *Bacillus* sp. SHC1 also produced the highest lignin peroxidase, at 209.30 U/L, on the fifth day of fermentation under the optimum conditions of pH 8 and 30 °C.
3. Strain *Ochrobactrum* sp. SHC2 showed the highest laccase enzyme production of the three selected bacterial strains in the present study. Although the production of manganese peroxidase and lignin peroxidase by strain *Ochrobactrum* sp. SHC2 was lower than *Bacillus* sp. SHC1 and *Leucobacter* sp. SHC3, *Ochrobactrum* sp. SHC2 showed the highest laccase enzyme at 10.21 U/L on the fifth day under the optimum conditions of pH 7.5 and 35 °C.
4. The *Leucobacter* sp. SHC3 strain was considered a novel ligninolytic bacteria isolate from palm oil plantation soil, as this is the first report of a *Leucobacter* sp. strain that can produce ligninolytic enzymes.

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