Isolation of Thermophilic Lignin Degrading Bacteria from Oil-Palm Empty Fruit Bunch (EFB) Compost

C M T Lai¹, H B Chua¹, M K Danquah¹² and A Saptoro¹
¹ Department of Chemical Engineering, Curtin University Malaysia, CDT 250, Miri 98009, Sarawak, Malaysia.
² Curtin Sarawak Research Institute (CSRI), Curtin University Malaysia, CDT 250, Miri 98009, Sarawak, Malaysia
E-mail: chua.han.bing@curtin.edu.my

Abstract. Empty Fruit Bunch (EFB) is a potential and sustainable feedstock for bioethanol production due to its high cellulosic content and availability in Malaysia. Due to high lignin content of EFB and the lack of effective delignification process, commercial bioethanol production from EFB is presently not viable. Enzymatic delignification has been identified as one of the key steps in utilising EFB as a feedstock for bioethanol conversion. To date, limited work has been reported on the isolation of lignin degrading bacteria. Hence, there is a growing interest to search for new lignin degrading bacteria with greater tolerance to temperature and high level of ligninolytic enzymes for more effective lignin degradation. This study aimed to isolate and screen thermophilic ligninolytic microorganisms from EFB compost. Ten isolates were successfully isolated from EFB compost. Although they are not capable of decolorizing Methylene Blue (MB) dye under agar plate assay method, they are able to utilize lignin mimicked compound – guaiacol as a sole carbon on the agar plate assay. This infers that there is no correlation of ligninolytic enzymes with dye decolourization for all the isolates that have been isolated. However, they are able to produce ligninolytic enzymes (Lignin peroxidase, Manganese peroxidase, Laccase) in Minimal Salt Medium with Kraft Lignin (MSM-KL) with Lignin Peroxidase (LiP) as the predominant enzyme followed by Manganese Peroxidase (MnP) and Laccase (Lac). Among all the tested isolates, CLMT 29 has the highest LiP production up to 8.7673 U/mL following 24 h of growth.

1. Introduction
In the past two decades, extensive research has been done on second generation bioethanol production using crop residues, saw-dust, wood chips and other agriculture wastes. Most of the lignocellulosic materials are made up from agriculture wastes (rice straw, wheat straw and sugarcane bagasse) and it is cost effective and abundantly available. Malaysia is the second largest supplier of palm oil which contributed about 41% of world’s supply on palm oil [1]. It has been reported that Malaysia produces 18.8 million tons of palm oil in 2012 [2]. Furthermore, production of palm oil is expected to increase up to 50 million tons per year in 2030 [1]. With the tremendous growth of palm oil production, it is predicted that 50 million tons of Empty Fruit Bunch (EFB) will be produced in 2030.

Moreover, EFB is a potential biomass material for biofuel production due to its high lignocellulosic content. Apart from that, EFB can be a promising source of renewable energy due to its abundance. In addition, bioethanol produced from EFB biomass is considered as second generation bioethanol (SGB). SGB is the ethanol derived from biomass wastes or materials of non-edible sources. Thus,
SGB is able to reduce competition of food for fuel production [3]. However, SGB production from EFB biomass is presently not viable due to high lignin content in lignocellulose structure. Lignin is a cross-linking structure (figure 1) which made up of aromatics polymers that associated with cellulose and hemicellulose as. High lignin content restricts the accessibility of enzymes involved in hydrolysis process which results in low sugar and bioethanol production. Thus, effective pretreatment process in degrading the lignin content is a vital aspect in bioethanol production from EFB as a bioethanol feedstock.

In recent years, biological method using microorganisms and enzymes has gained extensive interest in lignin pretreatment process due its cost effectiveness and environmental friendliness. Moreover, biological pretreatment process does not produce toxic compounds as much as physical and chemical process [4, 5]. Lignin peroxidase (LiP), Manganese peroxidase (MnP) and Laccase (Lac) are the major enzymes involved in lignin degradation process. To date, white rot fungi (WRF) such as Trametes versicolor, Trametes hirsuta, P. ostreatus and P. radiata are the common lignin degrading microorganisms that are capable of producing all the three major enzymes. However, ligninolytic enzymes from fungi has low stability under high working temperature, high pH condition and high substrate conditions [6, 7]. In fact, delignification process often involves high temperature up to 70 °C. Moreover, it is proven that bacterial enzymes are capable in degrading lignin and address the limitation of fungi [8].

In the current prospect, Paenibacillus sp., Bacillus sp., and Streptomyces sp. are the common bacteria that are involved in lignin degradation process [10-12]. It is reported that Bacillus sp., Pandoracea sp. and Streptomyces griseorubens have the capability in producing LiP, MnP and Lac enzyme. Among these strains, Bacillus sp. is able to degrade wide range of lignin model and lignin derivatives such as Guaiacylglycerol-β-guaiacyl ether (GGE) lignin model dimer and Kraft lignin up to 70% and 35.1% (Huang et al., 2013). However, most of the isolated strains are mesophiles. Furthermore, most of the bacteria isolates are isolated from soils, wheat straw, bagasse and industrial waste. The isolation study on thermophilic bacteria from EFB for lignin degradation is relatively scarce compared to mesophiles. Only a few researchers have attempted in isolating thermophilic bacteria from EFB. Ureibacillus terranus, Nocardioopsis sp., S. violaceorubidus and Streptomyces sp. were successfully isolated from EFB [13, 14]. However, those previous studies only demonstrated the presence of ligninolytic activities based on Remazol Brilliant Blue R (RBBR) decolouration without further examination on the LiP, MnP and Lac enzyme activities. Nevertheless, the studies above have shown the presence of thermophilic lignin degrading bacteria in the EFB compost. Thus, this study aims to isolate potential thermophilic lignin degrading bacteria from EFB compost and examine their ligninolytic enzyme activities.

Figure 1. Lignin structure [9]
2. Materials and methods

2.1. Sample collection
Young EFB (5 -10 day old) compost samples were collected from composting piles of a local oil palm mill. The compost was collected from a depth of about 20-30 cm from the compost surface and stored at 4 °C. The compost samples were used in the isolation of thermophilic ligninolytic bacteria.

2.2. Isolation of bacteria
For isolation of the bacteria, compost samples were re-enriched with Minimal Salt Media which contain 0.5 g/L of kraft lignin (MSM-KL) as sole carbon source. The MSM-KL medium contained (g/L): KL (0.5); K2HPO4 (4.55); KH2PO4 (0.53); CaCl2 (0.5); MgSO4 (0.5); NH4NO3 (5) with trace elements CuSO4 (0.002), FeSO4 (0.01); MnSO4 (0.001) and ZnSO4 (0.001)[7]. 5 g of EFB compost was added to 95 mL of sterile MSM-KL media placed in 250 mL Erlenmeyer flasks at 50 °C for 7 days. The solution was stirred vigorously and then allowed to stand for the compost to settle.

One ml aliquot was aseptically, serially diluted until a dilution of was obtained. 100 µL of diluted solution was plated on MSM-KL agar plate with 30 g/L of agar content. The plates were incubated at 50 °C for 7 days until colonies were formed. The isolated bacteria were plated onto fresh MSM-KL agar plates until pure cultures were obtained. Thirty four isolates were obtained and maintained in Tryptic Soy Agar (TSA) plates.

2.3. Screening of ligninolytic bacteria
The following three methods were used to screen the ligninolytic bacteria. (1) MSM-KL agar plate assays with 25 mg/L of Methylene Blue (MB) dye, 2.5 g/L Yeast extract, 2 g/L glucose; (2) TSA with 25 mg/L of MB, (3) 1 mM Guaiacol, a lignin mimicking compound was used as a carbon source in MSM-KL agar plate assays [12]. For plate assays, agar plates were incubated at 50 °C for seven days. Growth and decolourization of the agar was monitored daily.

2.4. Enzyme profile of the selected isolates
Ten isolates which showed good growth were selected and grown aerobically in 80 mL MSM-KL medium with 10 g/L glucose, 1.0 g/L yeast extract and addition of 2 mL inoculum in 250 mL Erlenmeyer flasks under submergence fermentation. Inoculum was prepared by inoculating one loopful of each selected isolates into 20 ml of Tryptic Soy Broth (TSB) and incubated at 50 °C for 16 h to obtain a final OD600 of 1.0. The MSM-KL medium with inoculum was incubated for 6 days and culture samples were removed at 24 h intervals. The un-inoculated MSM-KL medium was used as a control. The culture filtrate was centrifuged at 4,000 rpm for 20 min at 4 °C. The supernatant obtained after centrifugation was used for the enzyme assays.

2.4.1. LiP enzyme assay. The lignin peroxidase (LiP) activity were measured via the oxidation of veratryl alcohol to veratraldehyde at 310 nm. One 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 H2O2 (20 mM), which was freshly prepared daily and added to initiate the reaction [15]. The conversion to veratraldehyde is 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 molar extinction coefficient of \( \varepsilon_{310} = 93000 \text{ M}^{-1} \text{cm}^{-1} \).

2.4.2. MnP enzyme assay. Manganese peroxidase (MnP) activity was measured via the oxidation of guaiacol to a coloured product using UV-Vis spectrophotometer at 465 nm. 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 MnSO4 (1 mM), and 0.4 mL of supernatant of the sample [16]. 30 µL of H2O2 (0.1 mM)
which was freshly prepared daily and added to initiate the oxidation of guaiacol. The formation of the coloured product was measured in the 1 mL quartz cuvette at 465 nm wavelength. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 micromole of coloured product per minute under the assay conditions with a molar extinction coefficient of $\varepsilon_{465} = 12100 \text{ M}^{-1}\text{cm}^{-1}$.

2.4.3. Lac enzyme assay. Laccase (Lac) activity was assayed via oxidation of 2,2'-azinobis-(3-ethylbenzethiazoline-6-sulphonate) (ABTS) using method developed by Wolfenden and Wilson (1982). This enzymatic mixture made up of 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 of supernatant of the sample [17]. The formation of oxidized ABTS was measured in 1 mL quartz cuvette (1 cm light path) 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 $\varepsilon_{420} = 32000 \text{ M}^{-1}\text{cm}^{-1}$.

3. Result and Discussions

3.1. Isolation and screening of bacteria

Thirty-four bacteria isolates were successfully isolated using MSM-KL agar plate. In the screening process, none of the isolates showed decolourization on MSM-KL and TSA incorporated with MB as the lignin polymeric dye. However, there were bacteria growth in all of the plate assays. This result indicated that the agar medium could sustain the growth of these isolates in the presence of glucose. Although dye-based plate assay is a quick method used to screen for lignin degrading bacteria [18], but decolourisation of MB was not observed in the present study with dye-based plate assay. Similar observation was reported for Streptomyces psammoticus that is capable in producing LiP, MnP and Lac but has no decolourization ability on dyes such as Poly R and RBBR [12].

On the guaiacol plate assays, all ten isolates showed positive growth. These isolates were maintained in TSA medium. The characteristic features, morphology and size of the ten isolates’ colonies are stated in table 1. Although guaiacol is a common lignin model compound that could be degraded by LiP, MnP and Lac enzyme but no decolourization zone was observed around the colonies in any isolates. Formation of colonies on guaiacol plate assay in the absence of glucose has indicated the capability of the cultures to utilise guaiacol as the sole carbon source [12]. Table 1 summarised the growth ability of respective isolates in different agar plate assays. The bacteria colonies were visible with an average incubation time of 16 h and 24 h on TSA agar and MSM-KL agar that incorporated with MB dye respectively. The result in table 1 also revealed that isolate CLMT 20, CLMT 21 and CLMT 22 showed active growth regardless of any agar medium used. To date, several lignin degrading bacteria and actinomycetes such as Bacillus sp., Ureibacillus terrenus, Nocardiopsis sp., Streptomyces violaceorubidus and Streptomyces sp. were successfully isolated from oil palm plantation soils and EFB compost [7, 13, 14]. According to the result in table 1, all of the isolates are in rod shaped. Thus, it is believed that rod-shaped bacteria such as Bacillus sp., Pseudomonas sp., Leucobacter sp. and Ureibacillus terrenus could be the potential bacteria in these isolates.

3.2. Enzyme profile of the selected isolates

Ten isolates that showed positive growth on guaiacol plate assay were further screened for ligninolytic enzyme activities (LiP, MnP and Lac) using MSM-KL medium under submerged fermentation for 120 h. According to Chandra’s findings [20], bacteria isolates such as Citrobacter sp. and Citrobacter freundii utilize glucose and yeast extract as carbon and nitrogen source in the initial stage and subsequently use kraft lignin as co-metabolites. Thus, glucose and yeast extract was added to promote
the bacterial growth in ligninolytic enzyme production [21]. The enzyme activities for LiP, MnP and Lac were measured at the interval of every 24 h. Interestingly, all ten isolates showed positive result in production of all three main ligninolytic enzymes (LiP, MnP and Lac) simultaneously. It was found that LiP enzyme is the predominant enzymes followed by MnP and Lac for all the isolates. As shown in figure 2 to figure 4, LiP and MnP enzymes were found to be more active than Lac activity. Apart from that, high peroxidase enzyme (LiP and MnP) activity was detected during the first 24 h of incubation but gradually decreased until termination of experiment. In fact, most of the isolates achieved maximum LiP production at 24 h except for isolate CLMT 18 and CLMT 20 on LiP enzyme production.

Table 1. Characteristic features and growth of ligninolytic bacteria isolates in the following MSM-KL, TSA and

| Isolates | Morphology | size (μm) | Gram staining | Colour of colonies | MSM-KL + MB<sup>a</sup> | TSA + MB<sup>b</sup> | Guaiacol<sup>c</sup> |
|----------|------------|-----------|---------------|-------------------|-------------------------|---------------------|------------------|
| CLMT 5   | Rod shape  | 3.00, 0.67 | +ve           | Yellow            | +                       | ++                  | +                |
| CLMT 18  | Rod shape  | 2.33, 0.83 | +ve           | Yellow            | +                       | +++                 | +                |
| CLMT 19  | Rod shape  | 2.33, 0.67 | +ve           | Off-white         | +                       | ++                  | +                |
| CLMT 20  | Rod shape  | 2.00, 0.67 | +ve           | Yellow            | ++                      | +++                 | +++              |
| CLMT 21  | Rod shape  | 2.00, 0.50 | +ve           | Yellow            | ++                      | +++                 | ++               |
| CLMT 22  | Rod shape  | 2.67, 0.67 | -ve           | Yellow            | ++                      | +++                 | ++               |
| CLMT 23  | Rod shape  | 2.00, 0.83 | +ve           | Brown             | +                       | ++                  | +                |
| CLMT 28  | Rod shape  | 3.67, 0.50 | +ve           | Brown             | +                       | +                   | +                |
| CLMT 29  | Rod shape  | 2.33, 0.83 | +ve           | Off-white         | +                       | ++                  | +                |
| CLMT 34  | Rod shape  | 2.67, 0.50 | -ve           | Off-white         | +                       | ++                  | +                |

<sup>a</sup> MSM-KL + 25 mg/L MB dye + 2.5 g/L Yeast extract + 2 g/L glucose
<sup>b</sup> TSA + 25 mg/L MB dye
<sup>c</sup> MSM-KL + 1 mM guaiacol
+++; very fast growth (colonies visible after 12 hours)
++; fast growth (colonies visible after 18 hours)
+; slow growth (Visible colonies after 24 hours)
Figure 2. Enzyme profile of LiP enzymes activities for the selected 10 isolates

Figure 3. Enzyme profile of MnP enzymes activities for the selected 10 isolates
According to figure 4, low Lac enzyme activity was observed on the first 24 h, and it increased steadily with the maximum production at 72 h, and its activity was maintained towards the end of fermentation. The observation above is in agreement with other studies on lignin degrading bacteria such as *Comomanas* sp. B-9, *Pandoraea* sp. B-6, *B. subtilis* and *Klebsiella pneumonia* [22-24]. In fact, most of the isolates in the current study has a higher rate of enzymatic production since the maximum peroxidase production occurred after about 24 h duration of growth. Based on previous studies, peroxidase enzymes achieved maximum production in the first 96 h of incubation whereas Lac achieved its maximum production at the later stage. Both *B. subtilis* and *Klebsiella pneumonia* also achieved highest peroxidase (LiP and MnP) and Lac enzyme activities at 48 h and 96 h respectively. In comparison with the isolates obtained in this study, the rate of peroxidase enzyme production by mesophiles such as *B. subtilis* and *Klebsiella pneumonia* is relatively lower since longer period is required to achieve maximum peroxidase production. The phenomena of peroxidase enzyme being active in the early stage could be due to large lignin polymer molecules that are not able to traverse into the cell membrane in the initial stage. Thus, extracellular enzymes such as peroxidase enzymes are secreted externally to degrade lignin polymer compounds to smaller fragment during primary metabolism [22, 23]. Moreover, some strains such as *Peynopus cinnabarinus*, *P. sanguineus*, and *Phlebia radiata* produced maximum Lac enzymes under nitrogen-limited condition [25]. The carbon and nitrogen sources would be depleted towards the end of fermentation process. Hence, it is possible that the isolates in the present study preferred nitrogen-limited condition for Lac production, thus, Lac was secreted and reached its maximum production at about 96 h to 120 h.

In the present study, only isolate CLMT 29 showed a different trend on peroxidase and Lac enzyme production. Isolate CLMT 29 achieved maximum Lac enzyme activity at 24 h followed by declination till no Lac enzyme activity was detected from 72 h onwards. Moreover, CLMT 29 attained greatest LiP enzyme production at 8.7673 U/ml among all the tested isolates in this study. Apart from that, CLMT 29 also maintained a high level of LiP enzyme activity throughout the period of 24 h to 72 h but it has the lowest MnP and Lac enzyme activity compared to the other isolates involve.
Table 2. Highest LiP, MnP and Lac enzyme activities produced by selected isolates

| Isolates | LiP (U/mL) | Period (h) | MnP (U/mL) | Period (h) | Lac (U/mL) | Period (h) |
|----------|------------|------------|------------|------------|------------|------------|
| CLMT 5   | 3.9923     | 24         | 1.8759     | 24         | 0.5392     | 72         |
| CLMT 18  | 4.2495     | 48         | 1.6684     | 24         | 0.7475     | 96         |
| CLMT 19  | 5.8598     | 24         | 1.3726     | 24         | 0.4728     | 72         |
| CLMT 20  | 5.0323     | 72         | 1.5322     | 24         | 0.6606     | 96         |
| CLMT 21  | 4.3613     | 24         | 1.3790     | 24         | 0.4197     | 120        |
| CLMT 22  | 3.9140     | 24         | 0.6451     | 24         | 0.7443     | 120        |
| CLMT 23  | 7.0452     | 24         | 0.5533     | 48         | 0.7475     | 120        |
| CLMT 28  | 5.0658     | 24         | 0.6235     | 48         | 0.6357     | 72         |
| CLMT 29  | 8.7673     | 24         | 0.5150     | 24         | 0.2907     | 24         |
| CLMT 34  | 5.4572     | 24         | 0.6225     | 72         | 0.8178     | 72         |

Table 2 showed that isolate CLMT 29 and CLMT 23 produced greater LiP enzymes with maximum activities of 8.7673 U/mL and 7.0452 U/mL respectively. Other isolates produced comparative lower LiP enzyme activities which ranged from 3.9923 U/mL to 5.8598 U/mL. Result in table 2 revealed that CLMT 5, CLMT 18 and CLMT 20 have relatively high MnP enzyme production of 1.8759 U/mL, 1.6684 U/mL and 1.5322 U/mL respectively compared to other isolates. Apart from that, three isolates (CLMT 34, CLMT 18, CLMT 23) showed the highest Lac enzyme production with more than 0.7443 U/mL of enzyme activities. Based on the observation above, different isolates seemed to have different enzyme activities. The isolated bacteria isolates in the current study showed promising results since they are able to produce all major ligninolytic enzymes (LiP, MnP, Lac). Most of the lignin degrading bacteria reported only produced at most two enzymes (MnP with Lac or LiP with Lac) [22, 23, 26, 27]. To date, *Autochtonous Streptomyces* is the bacteria isolate that produced highest LiP activity of about 400 U/mL. The LiP activity of CLMT 29 obtained in this study is lower compared to *Autochtonous Streptomyces*. However, *Autochtonous Streptomyces* only produces LiP and Lac enzymes, and no MnP was detected. The presence of three major ligninolytic enzymes is vital since these enzymes worked synergistically in the lignin degradation process. So far, only several bacteria such as *B. subtilis*, *Klebsiella pneumonia* and *Bacillus* sp. SHC1 were capable in producing all three enzymes [7, 24]. Due to their capability, *Bacillus* sp. is recognised as a highly potential lignin-degrading bacteria.

Comparative study in table 3 showed that LiP enzyme activity in the current study is higher than the previous studies on mesophiles such as *B. subtilis*, *Klebsiella pneumonia* and *Bacillus* sp. SHC1 but lower than *Autochtonous Streptomyces*. The LiP enzyme activities for CLMT 29 is about 50 times lower than *Autochtonous Streptomyces* (400 U/mL). In fact, the LiP enzyme activity of CLMT 29 is also less active compared to *Stereum ostrea* that achieved 270 U/g of LiP enzyme activity [28]. Interestingly, the LiP activity of all the isolates obtained in this study is significantly greater than the reported study on rod-shaped bacteria *B. subtilis* and *Bacillus* sp. SHC1. The LiP activity for CLMT 29 is about 6 times higher than LiP enzyme activity obtained from B. subtilis [24]. Moreover, it is also comparable to some of the common lignin degrader white rot fungi such as *Pleurotus ostreatus* (7.250 U/mL) [29]. To date, there is no report on isolation of thermophilic lignin degrading bacteria with high LiP enzyme activity. This is the first report on production of LiP enzyme by thermophilic bacteria that was isolated from EFB compost.
Table 3. Comparison of LiP, MnP and Lac enzyme activities with previous studies on bacteria

| Isolates               | Maximum enzyme activities (U/mL) | Reference |
|------------------------|----------------------------------|-----------|
|                        | LiP | MnP | Lac | |
| *Comamonas* sp. B-9   | *ND | 2.9032 | 1.2500 | [22] |
| *Pandoraea* sp. B-6   | *ND | 2.2492 | 1.1206 | [23] |
| *Autotchtonous*       | 400 | *ND | 0.0950 |           |
| *Streptomyces*        |     |      |      |           |
| *B. subtilis*         | 1.5000 | 0.8000 | 3.4000 |           |
| *Klebsiella pneumonia* | 1.1000 | 0.6000 | 0.4000 | [24] |
| *Bacillus* sp. SHC1   | 0.1684 | 2.0097 | 0.0021 | [7] |
| CLMT 29               | 8.7673 | 0.5150 | 0.2907 |           |
| CLMT 5                | 3.9923 | 1.8759 | 0.5392 |           |
| CLMT 34               | 5.4572 | 0.6225 | 0.8178 |           |

Besides, the MnP activity for CLMT 5 (1.8759 U/mL) is also comparable to *Bacillus* sp. SHC1 (2.0097 U/mL) isolated from oil palm plantation soils. However, the MnP activities for the rest of the nine isolates are lower than *Bacillus* sp. SHC1. *Comamonas* sp. B-9 (2.9032 U/mL) achieved greater MnP production compared to CLMT 5 (1.8759 U/mL). This could be due to MnP being not the predominant enzyme for the isolated isolates in the current study. Apart from that, similar observation was observed on Lac enzymes. Among all the tested isolates, CLMT 34 (0.8178 U/mL) produced greatest Lac enzyme activity whereby its activity is higher than *Klebsiella pneumonia* (0.4000 U/mL) but it is about 4 times less active compared to *B. subtilis* (3.4000 U/mL). The variation in enzyme activities observed across different isolates implies that the production of enzyme activities could be isolate dependent and affected by the nutritional requirement in the fermentation medium.

4. Conclusions
Ten lignin thermophilic degrading bacteria isolates have been successfully isolated from the oil-palm EFB compost. This study highlights that these bacteria isolates have the capability in producing LiP, MnP and Lac simultaneously in the same fermentation medium. It was found that LiP is the predominant enzymes. In fact, most of the studies showed that MnP and Lac are the predominant enzymes in lignin degrading bacteria. Moreover, 90% of lignin is made up from non-phenolic compound that are not readily oxidised by MnP and Lac. Thus, LiP enzyme is highly useful in lignin degradation process as it has an outstanding ability in degrading non-phenolic aromatics compound. Additionally, most of the bacteria isolates obtained in this study achieved maximum enzyme production within a short period ranging from 24 h to 72 h. This preliminary study seemed to indicate that the isolated bacteria isolates could be useful in pretreatment lignin degradation process for bioethanol production from EFB. Future work on optimization of the fermentation medium and operational conditions can be performed to maximize the ligninolytic enzyme production by these isolates.

Acknowledgement
This research has been supported by Faculty of Engineering and Science, Curtin University Sarawak.

Nomenclature

| Abbreviation | Description                  |
|--------------|------------------------------|
| ABTS         | 2,2’-azinobis-(3-ethylbenzethiazoline-6-sulphonate) |
| EFB          | Empty fruit bunch            |
| GGE          | Guaiacylglycerol- β-guaiacyl ether |
| Lac          | Laccase                      |
| LiP          | Lignin peroxidase            |
References

[1] Lim J M H 2010 A case study on palm empty fruit bunch as energy feedstock SEGi Review 3 p 3-15
[2] Chang S H 2014 An overview of empty fruit bunch from oil palm as feedstock for bio-oil production Biomass Bioenerg. 62 p174-181
[3] Tye Y Y, Lee K T, Wan Abdullah W N and Leh C P 2011 Second-generation bioethanol as a sustainable energy source in Malaysia transportation sector: Status, potential and future prospects Renew. Sustainable Energy Rev. 15 p 4521-4536
[4] Agbor V B, Cicek N, Sparling R, Berlin A, and Levin D B 2011 Biomass pretreatment: Fundamentals toward application Biotechnol. Adv. 29 p 675-685
[5] Alvira P, Tomás-Pejo E, Ballesteros M, and Negro M J 2010 Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review Bioresour. Technol. 101 p 4851-4861
[6] Wang Y, Liu Q, Yan L, Gao Y, Wang Y, and Wang W 2013 A novel lignin degradation bacterial consortium for efficient pulping Bioresour. Technol. 139 p 113-119
[7] Abdul Rahman N H, Abdul Rahman N A, Abd Aziz S and Hassan M A 2013 Production of Ligninolytic Enzymes by Newly Isolated Bacteria from Palm Oil Plantation Soils
[8] Thambirajah J J, Zulkali M D and Hashim M A 1995 Microbiological and biochemical changes during the composting of oil palm empty-fruit-bunches. Effect of nitrogen supplementation on the substrate Bioresour. Technol. 52 p 133-144
[9] Bugg T D, Ahmad M, Hardiman E M and Singh R 2011 The emerging role for bacteria in lignin degradation and bio-product formation. Curr. Opin in Biotechnol. 22 p 394-400.
[10] Woo H L, Hazen T C, Simmons B A and DeAngelis K M 2014 Enzyme activities of aerobic lignocellulolytic bacteria isolated from wet tropical forest soils Syst.Appl. Microbiol. 37 p 60-67
[11] Naz S, Devtare S, Satapathy S and Gupta S 2015 Study of ligninolytic bacteria isolation and characterization from Dhamdha Agro Field of Bhilai-Durg Region IJERT 4 p 258262
[12] Niladevi K N and Prema P 2005 Mangrove Actinomycetes as the source of ligninolytic enzymes Actinomycetologica. 19 p 40-47
[13] Ting A S Y, Hermanto A and Peh K L 2014 Indigenous actinomycetes from empty fruit bunch compost of oil palm: Evaluation on enzymatic and antagonistic properties Biocatal. Agric. Biotechnol. 3 p 310-315
[14] Ting A S Y, Tay H, Peh K L, Tan W S and Tee C S 2013 Novel isolation of thermophilic Ureihacillus terrenus from compost of empty fruit bunches (EFB) of oil palm and its enzymatic activities Biocatal. Agric. Biotechnol. 2 p162-164
[15] Tien M and Kirk T K 1988 Lignin peroxidase of Phanerochaete chrysosporium Meth. Enzymol. 161 p 238-249
[16] Li X, Jia R, Li P and Ang S 2009 Response surface analysis for enzymatic decolorization of Congo red by manganese peroxidase J. of Mol. Catal. B Enzym. 56 p 1-6
[17] Wolfenden B S and Willson R L 1982 Radical-cations as reference chromogens in kinetic studies of ono-electron transfer reactions: pulse radiolysis studies of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) J. Chem. Soc. Perkin Trans. 2. p 805-812
[18] Bandounas L, Wierckx N, de Winde J and Ruijssenaars H 2011 Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential BMC Biotechnol. 11 p 1-11
[19] Machado K M G, Matheus D R and Bononi V L R 2005 Ligninolytic enzymes production and Remazol brilliant blue R decolorization by tropical brazilian basidiomycetes fungi Braz. J. Microbiol. 36 p 246-252
[20] Chandra R and Bharagava R N 2013 Bacterial degradation of synthetic and kraft lignin by axenic and mixed culture and their metabolic products J. Environ. Biol. 34 p 991-999
[21] Shi Y, Chai L, Tang C, Yang Z, Zhang H, Chen R, Chen Y and Zheng Y 2013 Characterization and genomic analysis of kraft lignin biodegradation by the beta-proteobacterium Cupriavidus basilensis B-8 Biotechnol Biofuels 6 p 1-14
[22] Chen Y H, Chai L Y, Zhu Y H, Yang Z H, Zheng Y and Zhang H 2012 Biodegradation of kraft lignin by a bacterial strain Comamonas sp. B-9 isolated from eroded bamboo slips J. Appl. Microbiol. 112 p 900-906
[23] Shi Y, Chai L, Tang C, Yang Z, Zheng Y, Chen Y and Jing Q 2013 Biochemical investigation of kraft lignin degradation by Pandoraea sp. B-6 isolated from bamboo slips Bioprocess Biosyst. Eng. 36 p 1957-1965
[24] Yadav S and Chandra R 2015 Syntrophic co-culture of Bacillus subtilis and Klebsiella pneumonia for degradation of kraft lignin discharged from rayon grade pulp industry J. Environ. Sci. 33 p 229-238
[25] Elisashvili V, Kachlishvili E and Penninckx M 2008 Effect of growth substrate, method of fermentation, and nitrogen source on lignocellulose-degrading enzymes production by white-rot basidiomycetes J. Ind. Microbiol. Biotechnol. 35 p 1531-1538
[26] Chen Y, Chai L, Tang C, Yang Z, Zheng Y, Shi Y and Zhang H 2012 Kraft lignin biodegradation by Novosphingobium sp. B-7 and analysis of the degradation process. Bioresour. Technol. 123 p 682
[27] Buraimoh O M, Amund O O and Ilori M O 2015 Kraft lignin degradation by Autochtonous Streptomyces strains isolated from a tropical lagoon ecosystem J. Microbiol. Biotechnol. Food Sci. 5 p 248
[28] Usha K Y, Praveen K and Reddy B R 2014 Enhanced production of ligninolytic enzymes by a mushroom Stereum ostrea Biotechnol. Res. Int. 2014 p 9
[29] Kheiralla Z, El-Din S, Malek S and Aziz D 2013 Optimization of cultural conditions for lignin peroxidase production by Phanerochaete chrysosporium and Pleurotus ostreatus. Academia J. Biotechnol. 1 p 87-95