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ISOLATION AND IDENTIFICATION OF LOCALLY ISOLATED LIGNIN DEGRADING BACTERIA

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Abstract: Lignocellulosic biomass is a renewable and abundant resource with great potential for bioconversion to value added bioproducts. In Malaysia, this biomass become a major problem to the environment since most of these materials come from the waste of palm oil industry. However, the conversion of the lignocellulosic material becomes a hurdle due to lacking of biocatalysts that can overcome the biorefining process. Therefore, this study is aimed to isolate and identify locally isolated bacteria that are able to degrade lignin. Twenty two bacterial strains were isolated from decayed plants in Agro Park, Universiti Malaysia Kelantan Jeli Campus, Kelantan. Out of twenty two isolates, eight potential strains designated as ZA1, ZA2, ZA32, ZA42, ZA5, ZA71, ZA72 and ZA9 were found capable to effectively degrade the lignin through screening on selective agar plate contaning alkaline lignin as sole carbon source. Subsequently, partial sequence of 16S rRNA gene identified strains ZA1, ZA32 and ZA72 as *Klebsiella* sp., isolates ZA2, ZA42, ZA5 and ZA9 as *Enterobacter* sp. and isolate ZA71 as *Bacillus cereus*. From phylogenetic tree analysis, isolate ZA1 was found to be closely related to isolate ZA72 and isolate ZA2 was found to be closely related to isolate ZA42.

KEYWORDS: Lignin-degrading bacteria, identification.

Introduction

The expansion of agro-industry activity in Malaysia has led to an accumulation of agro industrial wastes. About 4.49 million ha of land is planted with palm oil trees throughout Malaysia. In the process of extracting the palm oil from the fruit, various solid wastes are produced which include empty fruit bunch (OPEFB), seed shells and fibre from mesocarp. Approximately 15 million tonnes of OPEFB are generated annually throughout Malaysia (Rahman *et al.,* 2006). In practice, the waste is burned which subsequently will cause environmental pollution. In Malaysia, this biomass become a major problem to the environment since most of these materials come from the waste of palm oil industry. Hence, there is an urgent need for a sustainable waste management system to tackle these wastes.

Lignocellulosic materials are composed of cellulose, hemicelluloses and lignin. Cellulose and hemicelluloses are polysaccharide that can be hydrolyzed to produce simple sugar. Many factors such as lignin, cellulase crystallanity, degree of polymerization limit the digestability of the hemicelluloses and cellulose (Zhao *et al.,* 2011). It is a major carbon source and has significant concentration of simple carbohydrates. A good understanding of these chemical constituents of the cell wall components is important to develop a good mechanism for the conversion of these constituents into more value added products such as low price chemicals (eg; xylitol, xylose), biofibres, biopulps and ruminant feed. The conversion of these lignocellulosic materials into value added end products becomes a hurdle due to lacking of biocatalysts that can overcome the biorefining process (Yang *et al.,* 2011).

There are high potential of lignin-degrading bacteria and the chacterization of lignin degrading enzymes may give potential benefits for biofuel production from lignocellulosic material. Previously, white rot fungi was considered to be the primary lignin degrader. However, the ability to degrade lignin by bacteria may provide

a lot of advantages compared to fungi such as the ability adapted better in anaerobic conditions (Huang *et al*., 2013), higher potential due to its environmental adaptibility and biochemical versatility (Abd-Elsalam, 2009).

The conversion of lignocellulosic materials to its derivatives using whole microbial cells or enzymes has been suggested as an economically feasible process and potential to reduce the use of fossil fuels and reduce economic pollution. The advantages of this process over chemical pretreatment includes low energy requirement, mild reaction, high substrate specificity, high yield of products and high hydrolysis efficiency. However, the pretreatment process conditions must be tailored to the specific chemical and structural composition of the various and variable sources of lignocellulosic materials (Mosier *et al.,* 2005).

Therefore, this study is aimed to screen, isolate and identify novel bacteria producing lignin degrading enzymes that have the potential to degrade lignocellulosic biomass from decayed plants.

Methodology

Bacterial Sources

Decayed plants from UMK Agro Park area were collected as microbial sources. The samples were inoculated in 10 ml Nutrient Broth (NB) medium and incubated overnight at 32 °C. The cultures were serially diluted and plated on Nutrient Agar (NA) for isolating bacterial strains. The pure bacterial strains were isolated by repeated sub cultures and the pure cultures were maintained on nutrient agar plates and stored at 4 °C, while the stock was maintained in 15% glycerol at -80 °C.

Screening of Lignin Degrading Bacteria

Each isolates was further screened on selective agar containing malt extract agar (48 g/L), alkaline lignin (4 g/L), calcium carbonate (2 g/L) and toluidene blue (0.025 g/L). The agar plates were incubated at 32 °C for 24 hours. The plates were monitored for the growth and the development of decolorization zones.

Bacterial Identification

The positive isolates were then identified by 16S rRNA gene identification. The genomic DNA was extracted using DNeasy Tissue Handbook (Qiagen, Germany) according to manufacturer's instructions and the 16S rRNA gene was amplified by PCR using the universal primers (Rahman *et al*., 2007); forward primer: 5'-GAG TTT GAT CCT GGC TCA-3' and reverse primer: 5'-CGG CTA CCT TGT TAC GAC TT- 3'. Amplification reactions were performed in a total volume of 50 µl. The reaction mixtures contained 5 µl DNA, 6 µl of each forward and reverse primer, 25 µl 2X *Taq* Master Mix and 8 µl sterile distilled water. After 4 min pre-denaturation at 94 °C, 30 cycles of PCR including 1 min at 94 °C (denaturation), 1 min at 58 °C (annealing) and 1 min at 72 °C (extension) were performed. This was followed by one cycle of 7 min at 72 °C and held at 4 °C. The PCR products were examined by electrophoresis and detected using ethidium bromide fluorescence. QIAquick PCR Purification Kit (Qiagen, Germany) methods were used for the purification of PCR product and the purified PCR product was sent for sequencing. The sequence for DNA homology was matched with the Genebank database that was available at http://www.ncbi.nlm.nih.gov/BLAST/).

Phylogenetic Tree Analysis

The multiple sequence alignments analysis between the isolates and other bacteria strains were conducted using Clustal Omega (http://www.ebi.ac.uk). Phylogenetic tree was constructed based on the multiple sequence alignment analysis and displayed using TreeView program.

Results and Discussion

Screening of Lignin Degrading Bacteria

Based on qualitative screening on the selective agar, 8 out of 24 isolates showed positive results (Figure 1). The decolourization of toluidine blue dye indicates the positive strain for lignin degradation. The positive isolates were designated as ZA1, ZA2, ZA32, ZA42, ZA5, ZA71, ZA72 and ZA9.

Bacterial Identification

The rRNA sequence comparisons help to construct a universal tree of life, dividing all life on earth into three equidistant domains namely Eukarya, Bacteria and Archea (Woese, 1998). From the analysis, isolates ZA1, ZA32 and ZA72 were identified as *Klebsiella* sp., isolates ZA2, ZA42, ZA5 and ZA9 were identified as *Enterobacter* sp. and isolate ZA71 was identified as *Bacillus cereus*.

Phylogenetic Tree Analysis

The phylogenetic tree was constructed based on comparison of 16S rRNA sequences of the positive strains with other bacterial strains that were extracted from GeneBank database (http://www.ncbi.nlm.nih.gov). Ten of 16S rRNA sequences from different strains were compared. All sequences were aligned with Clustal Omega and phylogenetic tree was constructed.

The phylogenetic tree in Figure 2 demonstrated the linkage of the eight positive strains with other ten bacteria strains. From the phylogenetic tree of 16S rRNA sequences, *Klebsiella* sp. ZA1 was found to be closely related to *Klebsiella* sp. ZA72 and *Enterobacter* sp. ZA2 was found to be closely related to *Enterobacter* sp. ZA42. Obtained result also revealed that the sequence of *Klebsiella* sp. ZA32 showed the highest similarity with *Klebsiella* sp. SZH11(GU384262), and the sequence of *B. cereus* ZA71 showed the highest similarity with *B. cereus* cr-50 (JF895490). Meanwhile, *Enterobacter* sp. ZA5 was found to be closely related to *Enterobacter* sp. B25(2012) (JX941520) and *Enterobacter* sp. ZA9 was found to be closely related to *Enterobacter* sp. DC6 (HM625774). The phylogenetic tree analysis also indicated that all of these positive strains except *B. cereus* ZA71 were phylogenetically distant from other bacterial species such as *Aeromonas* sp. H1 (AM179893), *Pseudomonas stutzeri* SP1402(U26418), *Xanthomonas* sp. EB5(HF566369) and *Agrobacterium tumefaciens* RV3(AJ389903).

Gene identification using 16S rRNA has been done for more than a decade to analyse evolutionary relationships between organisms. Ribosomal RNAs are ancient molecules, functionally constant, universally distributed and moderately well conserved across broad phylogenetic distance.

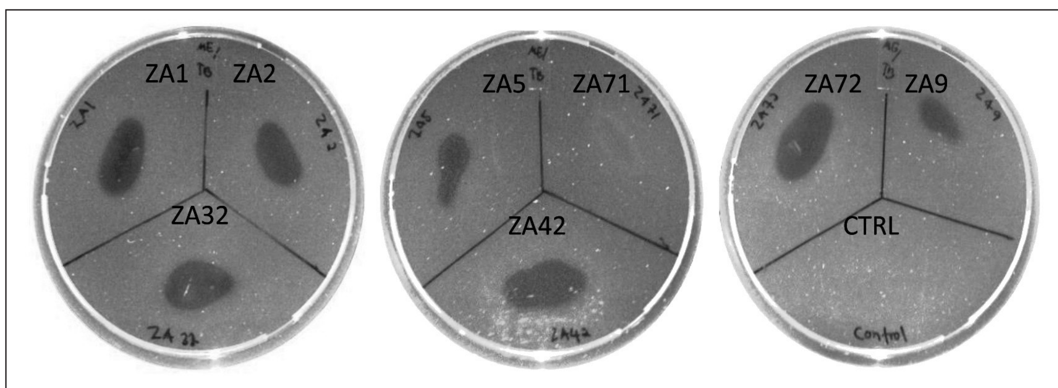

Figure 1: Decolourization Zones in Toluidine Blue-containing Plates After 24 h of Incubation. ZA1, ZA2, ZA32, ZA42, ZA5, ZA71, ZA72 and ZA9: bacterial isolates; control: no bacteria

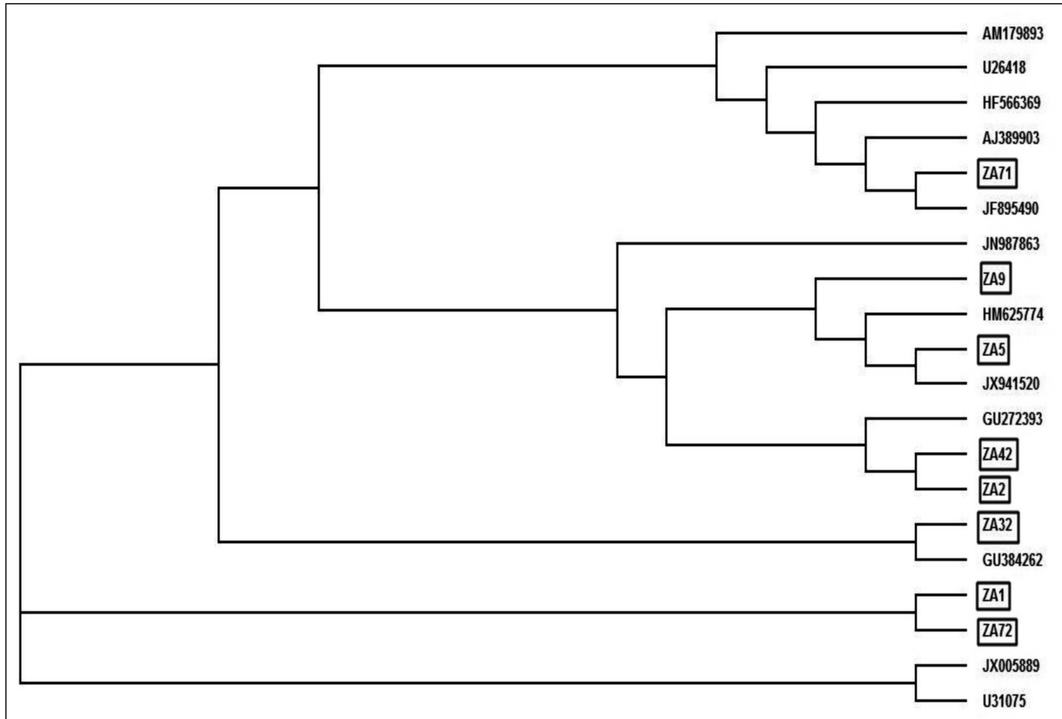

Figure 2: Phylogenetic Tree Showing the Relationship between Bacterial Strains ZA1, ZA2, ZA32, ZA42, ZA5, ZA71, ZA72 and ZA9 to Other Bacteria Strains

*AM179893 (*Aeromonas* sp. H1), U26418 (*Pseudomonas stutzeri* SP1402), HF566369 (*Xanthomonas* sp. EB5), AJ389903 (*Agrobacterium tumefaciens* RV3), ZA71 (*Bacillus cereus* ZA71), JF895490 (*Bacillus cereus* cr-50), JN987863 (*Enterobacter* sp. FF3), ZA9 (*Enterobacter* sp. ZA9), HM625774 (*Enterobacter* sp. DC6), ZA5 (*Enterobacter* sp. ZA5), JX941520 (*Enterobacter* sp. B25(2012)), GU272393 (*Enterobacter* sp. WP2ME), ZA42 (*Enterobacter* sp. ZA42), ZA2 (*Enterobacter* sp. ZA2), ZA32 (*Enterobacter* sp. ZA32), GU384262 (*Klebsiella* sp.SZH11), ZA1 (*Klebsiella* sp. ZA1), ZA72 (*Klebsiella* sp. ZA72), JX005889 (*Klebsiella* sp. SO22-4051), U31075 (*Klebsiella* sp. ZMMO).

In this study, the isolated strains have been identified as *Klebsiella* sp, *Enterobacter* sp and *B. cereus* according 16S rRNA gene identification. Lignin degradation by the bacteria was observed to have the ability to breakdown the lignin in the media containing lignin as sole carbon source. These isolates formed clearing zones on the media containing dye in this case, toluedine blue as colour indicator. *Bacillus* sp. had been reported to be a potential species to degrade lignin by Abd-Elsalam (2009) and Bandounas *et al.,* (2011). Besides that, *Achromobacter* sp., *Flavobacterium* sp., *Pseudomonas* sp. as well as nitrifying bacteria such as *Nitrobacter* and *Nitrosomonas* has been reported to have the ability to degrade lignin (Sharafi-Yazdi, 2001).

Previously, the UMK Agro Park was a tropical forest, then it was used for rubber plantation for a period of 20 years after which it is now under Agro Park. Still, it carries a lot of biodiversity in microorganisms living in the soils. According to Parton *et al.,* (2007), microorganisms isolated from rainforest soils have been identified as the fastest decomposers of the plant biomass compared to other.

There are clear evidances that four enzymes are actively involved in lignin degradation namely lignin peroxidase (LiP) [E.C.1.11.1.14], Mn-peroxidase (MnP) [E.C.1.11.1.13], versatile peroxidase (VP) [EC1.11.1.16] and laccase (Lac) [E.C.1.10.3.2]. In addition, other enzymes such as H_2O_2 producing enzymes, glyoxaloxidase

(GLox) and aryl alcohol oxidase (AAO) [EC 1.1.3.7] have their roles to degrade lignin. Furthermore, these enzymes can be used in wider field such as in pulp and paper industry, textile industry, bioconversion, feed, food processing, chemical industry and biosensors.

Conclusion

This study identified 3 bacterial isolates which can be used to degrade lignin from decayed plants more effectively which in bioconversion of lignocellulosic biomass into other value added end products such as as biofuel, sugar productions and management of waste to wealth.

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