

IDENTIFICATION AND EXPRESSION OF LIGNINASE ENZYMES FROM TROPICAL ASIA WOOD INSECT FOR AGRO-PULP BIODELIGNIFICATION: A THEORETICAL FRAMEWORK

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Abstract. Current pulp-processing in pulp and paper based industries are inefficient in removing the lignin as this compound is recalcitrant towards degradation. Transitioning from conventional pulping process into bio-delignification through utilisation of ligninase enzymes is one of the alternatives to improve the ability to fully utilize all components of wood to produce high quality fibres. Extensive research efforts have been focused on increase the production of ligninase enzymes from white rot fungi as a whole organism for industrial applications. However, enzymes activity produced from fungi are rather low as lignin modification is a secondary metabolism in which the enzyme only be expressed under particular conditions. Using genetic manipulations to incorporate genes associate for delignification isolated from different organisms such as tropical Asian wood-feeding insect into bacteria expression system will allow rapid enzyme production. This theoretical framework aims to produce an enzyme with high ligninase activity that will be used for removal of lignin during pulp-processing. These enzymes are thought to be more economically efficient in degrading lignin and involves less use of chemicals thus make this processing more environmentally friendly.

Introduction

Conventional pulp-processing are inefficient in removing lignin as this compound is resistant towards degradation. The conventional pulp processing; chemical processing require massive amount of chemicals while in mechanical processing huge energy consumption is required which contribute in producing greenhouse gases emission and other pollutants such as chlorinated wastewater from the processing reactor [1]. Interestingly, researchers exploring numbers of potential agro-wastes with low lignin content to reduce the usage of chemicals [2], [3]. But the pulping process still required large amount of chemicals [2], [3]. One of the alternatives proposed that is cost-effective in removing lignin and green to the environment is through bio-delignification. Instead of using a lot of chemicals or mechanical equipment to process the pulp, bio-delignification involve the use of enzymes such as ligninase to degrade the lignin compound. Lignin is the second abundant aromatic compound in plant cell wall that is wrapping around the cellulose and hemicelluloses [4]. The compound is thought to be unusual due to its heterogeneity and lack of a defined primary structure[5]. The arrangement of lignin are complex and undefined hence it is recalcitrant against degradation by most of organisms [5]. In nature, lignin is slowly removed by ligninase produce by compost organisms such as white rot fungi [6] and bacteria from the soil[7]. Apart from fungi and bacteria, it is known that the wood-feeding insects such as termites and longhorn Asian beetle also have the ability to degrade lignin by expressing enzymes degrading

lignin [8], [9]. Due to this ability, utilising ligninase in biodelignification hold huge potential to solve the issue of removing lignin during the pulp-processing.

Recent researches have been focused on increasing the production of ligninase from the white fungi in both solid-state fermentation (SSF) and submerged fermentation (SmF). However, the process of using fungi as a whole organism in this fermentation proved to be difficult to regulate due to its sensitivity to the shear force that are presence in the bioreactor and the agitation which inhibit ligninase production and activity [10]. Furthermore, the enzymes activity seems to be very low as the maximum enzymes activity recorded approximately between 8-16 days [11]. This is because in fungi, lignin degradation is a secondary metabolism by which the enzyme only be expressed under particular conditions such as carbon and nitrogen nutrient deprivation or heat stress conditions[12, 13]. Therefore, various metabolic parameters need to be taken into account and optimization of these parameters is difficult. Rather than using the whole organisms to carry out the process, isolating the enzymes to do the specific bio-process would be more convenient in a large scale process.

Current advancement in technology of cloning and sequencing have allowed manipulation of DNA for rapid production of targeted genes and subsequently expressing the enzymes of interest [11]. Expressing recombinant ligninase enzyme from the insect into bacteria expression system will allowed rapid production of this enzyme with high ligninase activity [11]. Therefore, this study is fundamentally interested in investigating the potential of manipulating DNA coding region for ligninase enzymes isolated from wood-feeding insects for bio-delignification of non-wood pulp materials.

Research Methodology

Database searches and analysis of DNA sequence for genes encoding ligninase. The DNA sequences encoding for ligninase are identified through the NCBI database. Using a software package for bioinformatics analysis (CLC Sequence viewer 7) to analyse DNA sequences by comparing the nucleotide sequences from available organism (*P. Chrysosporium*, *P. sordida*, *T. versicolour* and *P. radiata*) to design primer at conserved region for ligninase coding region (CDS). Primers are designed for all peroxidase: forward and reverse primers for; i) Manganese Peroxidase (MnP), ii) Lignin Peroxidase (LiP), iii) Versatile Peroxidase (VP), iv) Dye-Decolorizing peroxidase (DyPs) and v) Laccase (Lac) using NCBI Primer-BLAST software tools. These primers are used in identifying the corresponded gene from the fungi, termites and sago worm (*Rynchophorus ferrugineus*).

DNA Isolation, Quantification and Identification. The DNA is isolated from our organisms of interest: *Rhynchophorus ferrugineus* (sago worm), termites and fungi. These samples are collected around Batu Pahat, Johor. The DNA extraction is conducted using CTAB DNA extraction protocol [14]. DNA from organism fungi *P. Chrysosporium* also will be isolated and acts as a positive control for the primers. DNA quality is checked using spectrophotometer 230/260 nm absorbance ratio (ratio >1.8 is considered as good quality of DNA). The quantification of isolated DNA is quantified using spectrophotometer and known concentration lambda marker. The identification of corresponded ligninase genes in the DNA extraction involves the use of PCR reaction using the designated forward and reverse primer. In order to validate the genes, the PCR product is send for sequencing.

Cloning and Transformation. Cloning and transforming the recombinant plasmid into suitable bacteria expression system allows the gene to be translated into a functional enzyme protein. Bacteria expression system is thought to be rapid and cheap which is excellent in producing enough amount of protein enzyme for large scale industrial applications.

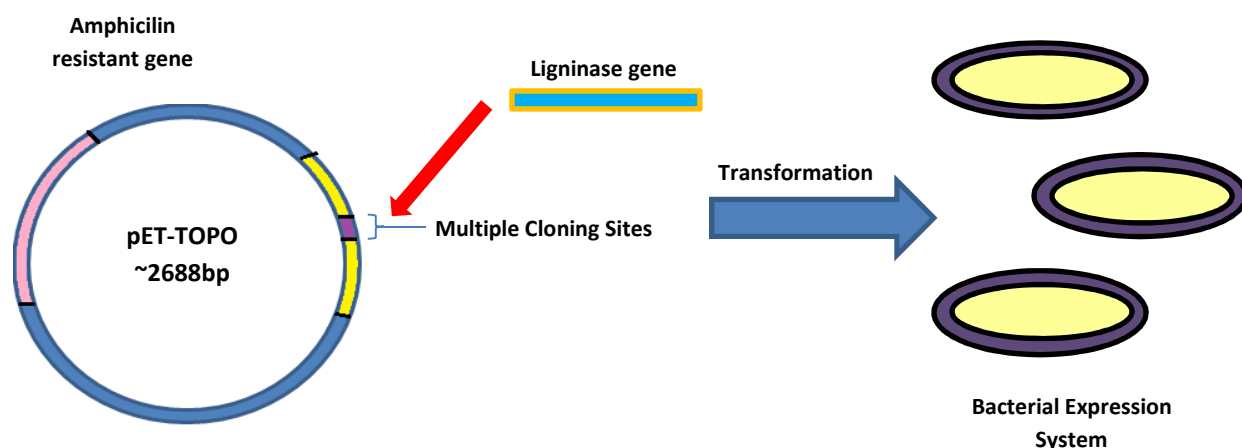


Figure 1: An illustration of cloning and transforming for ligninase gene into bacterial expression system for robust enzyme expression.

Enzyme Extraction and Purification. The expressed enzymes can be purified through protein purification chromatography technique. The expressed protein is tagged together with histidine allowed the expressed protein can specifically obtain through His-spin column chromatography column (IMAC). The purified protein is validated via protein sequencing using MALDI-MS/MS technique.

Enzyme Activity Assay. The activity of the purified enzyme is measure via enzyme activity assay to determine the efficiency of this enzyme in removing the lignin content in pulp. This qualitative assay involve the use of azure B dye as most of extracellular lignin peroxidase enzymes have a potential to degrade a wide range of complex aromatic dye- compound. The enzymes activity and the efficiency in terms of delignifying the pulp from the insect is compared with the chemical and mechanical processing to suggest better ligninase enzymes for large-scale industrial applications.

Expected Outcomes. Generally the outcomes of this research can be translated into many industry applications. This will include pulp and paper-based industries, biofuel production and feedstock area. Throughout this research, the expected detailed outcomes would be:

1. 5 sets of designated primers (forward and reverse primers) that corresponding to the genes of interest (LiP, MnP, VP, DyPs and Laccase) are obtained.
2. DNA sequences that encode lignin degrading or modifying enzymes from the wood feeding insects are identified.
3. Optimization conditions for stable expression of recombinant ligninase in bacterial expression system are constructed.
4. Extraction of purified recombinant ligninase enzymes from bacterial cell cultures is obtained.
5. Protein sequences that encode for the expressed ligninase enzymes are validated.
6. Enzyme activity and the efficiency to remove lignin content from the waste substrate are identified.

Conclusion

It is possible to isolate and identify genes encoding for ligninase other than fungi. Using molecular tools, the identified genes can be expressed using bacterial expression system for more rapid enzyme production that is suitable for large-scale industrial application. Furthermore, the enzymes isolated from insects could potentially have greater enzymes activity in terms of delignifying in the pulp compared to the chemical and mechanical processing. The final product from this research (ligninase enzymes) can be applied into various industrial processing including paper-pulp processing, biofuel and feedstock for effective industrial process with low pollution impact to the environment and promoting green technology that are eco-friendly processing.

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