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EXPLORING NOVEL MICROBIAL LIGNOCELLULOSIC ENZYMES FROM INDONESIA PEATLAND AND HERBIVORES FOR CONVERSION OF OIL PALM EMPTY FRUIT BUNCH TO BIOFUEL

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SUMMARY

As the world's number one producer of palm oil, the palm oil industry in Indonesia produces more than 30 million metric tonnes of empty fruit bunches (EFB) every year. Currently, most of these biomass residues have little value or go to waste. These biomass residues can be converted into a number of value-added products, such as biofuels. On the other hand, a large part of Indonesian microbial biodiversity remains unknown to science due to minimum research funds and capabilities in Indonesia. The objective of this study is to explore Indonesia's biodiversity and to find novel enzymes that can improve the efficiency of converting Indonesia's abundant biomass residues into biofuels. We target microbial communities living in peat swamp forests and herbivore guts, that are likely sources of novel lignocellulose-degrading enzymes. Bioinformatic and metagenomics are used to identify and screen for lignocellulosic enzymes that can improve the bioconversion process from Indonesia's microbial biodiversity. Culturable microbes are isolated, purified, identified and screened for active enzymes using synthetic substrate and oil palm empty fruit bunch hydrolysate; and for the ability to convert hydrolysate to biofuel. The long term goal of the study is to promote the development of biorefineries by identifying new enzymes that can lower the cost of bioconversion, and new microbes that can facilitate the production of new products from biomass residues.

Keywords: Biodiversity, biomass, residue, metagenomic, biorefinery,

INTRODUCTION

Lignocellulose is the most abundant form of biomass, with an annual world production of around 170 billion metric tons (Amidon & Liu, 2009). Indonesia alone, as the world's number one producer of palm oil produces more than 30 million metric tons of empty fruit bunches biomass every year. Currently, most of these biomass residues have little value or go to waste. These biomass residues can be converted through biochemical pathways into a number of value-added products, such as liquid fuels, chemicals, electricity, and fibers, that can substitute for products currently derived from fossil fuels. The conversion of lignocellulosic biomass into this range of value-added products is known as the biorefinery concept. However, woody plant biomass that is composed mainly of cellulose, hemicelluloses and lignin is recalcitrant to natural degradation. Lignin is essentially the largest contributor to biomass recalcitrance (Strachan *et al.*, 2014). It is the cement that binds plant materials together and is the most abundant aromatic component of higher plant cell walls, such as woody plants including ferns (T K Kirk & Farrell, 1987). Biomass recalcitrance has made it a challenge for research especially in microbial breakdown.

Conversion of biomass into simple sugars requires a large amount of enzymes to accelerate the degradation process. Consequently, this use of a high amount of enzymes increases the cost of the bioconversion process. Over the past 10 years, intense research efforts have focused on trying to lower the cost of enzymes in the bioconversion process. There are generally three strategies that have been proven effective in lowering the enzyme costs: reducing the cost of enzyme production, improving the efficiency of individual enzymes through genetic and protein engineering, or creating a more efficient enzyme mixture by enhancing the synergistic activities of the individual enzyme components in the mixture. Arguably, most of the improvements in the efficiency of enzyme mixtures, and the resulting decrease in the cost of using enzymes, have been achieved through the last strategy. In recent years, many new enzymes with novel activities have been identified and characterized to have synergistic effects with classic cellulolytic enzyme mixtures during the bioconversion process.

Both Indonesia and Malaysia are countries of mega-biodiversity. Our research aims to develop Indonesia‘s capacity in biodiversity research to facilitate exploration of Indonesia's biodiversity both using metagenomic and culturable microbes in order to identify new enzymes and microbes that can enhance the efficiency of the bioconversion process as well as to build a culture collection to facilitate ex-situ conservation of Indonesia‘s

microbial diversity. The long term goal of the study is to promote the development of biorefineries by identifying new enzymes that can lower the cost of bioconversion, and new microbes that can facilitate the production of new products from biomass residues.

The microbial diversity of Indonesia's peat swamp forests and of herbivore guts are chosen for our study as potential environmental pools that harbor lignocellulosic enzymes. Peat is partially decomposed biomass material that gets accumulated over years and is typically enriched in lignin and humic acid (Factors, 2015). The high proportion of lignin in peat may naturally promote the selection of microbial communities that can degrade or modify lignin. On the other hand, buffaloes, unlike cows, are known to be able to thrive on a diet of low quality feed (Bilal *et al*., 2006). Therefore, buffaloes likely harbor gut microflora that can efficiently break down cellulose and hemicelluloses, enabling the herbivores to extract nutrients from this low quality feed. Therefore, microbial communities thriving in peat and buffalo gut are ideal sources to find novel enzymes that can improve the efficiency of the bioconversion process.

Both bioinformatics and metagenomics approaches will be used to identify new enzymes from the microbial diversity in these two ecosystems as these approaches allow for an assessment of the total genetic diversity in an environmental sample. Bioinformatics approach is used to evaluate and identify new enzymes that can improve the bioconversion process from Indonesia's biodiversity in peat soil and buffalo manure. Metagenomics are used to explore the total genetic diversity of enzymes present in an environmental sample. In addition, culturable microbes are also isolated and screened for active enzymes using synthetic substrates such as pure cellulose and oil palm empty fruit bunches as a representation of a real lignocellulosic biomass.

METHODS

Field sampling and microbial isolation

Environmental samples are gathered from two different environments: peat swamp and herbivore manures. Buffalo and cow manure were collected from PT. Sumber Alam Ciapus (SAC), Bogor. SAC center breeds buffaloes and cows with the focus on commercial breeding of spotted buffaloes. Eleven fresh manure samples from seven buffaloes and four cows were collected; pH and temperature of the manure were measured. Each sample was split into three different categories: crude samples, metagenomic analysis, and culturable microbes. All three are preserved at -80ºC. To isolate culturable microbes, environmental sample is dissolved in phosphate buffer saline solution and 50µL slurry was plated on three types of media, potato dextrose agar (PDA) with chloramphenicol, rose bengal chloramphenicol agar (RBCA), and 1/10 strength nutrient agar (NA) with cycloheximide. These media allow isolation of filamentous fungi, yeast and bacteria, respectively. The plates are incubated at room temperature (24ºC) until visible colonies are observed. Each different colony is purified on pure PDA without chloramphenicol and preserved at -80ºC for further use. Filamentous fungi, yeast and bacteria are identified to species level by ribosomal sequencing using standard methods (Golomb *et al*., 2013; Kumar & Shukla, 2005; Weisburg *et al.*, 1991). For the yeast species, the taxonomy term "~~a~~ff." indicates that the strain belongs to a novel species that is most closely related to the species (Garay *et al.*, 2016). Peat swamp sampling and sample processing are currently ongoing.

In addition, both oil palm EFB and peat will also be used to enrich for microbes that can degrade lignin. EFB and peat will be added to the growth media as carbon sources at concentrations of 10 g/ml. Microbes will be grown at 50^{o}C and growth will be monitored daily. After sufficient growth has been achieved, supernatants will be collected and analyzed for biomass conversion, enzymatic activities, and protein content. In addition, the microbes will be collected along with the biomass pellet.

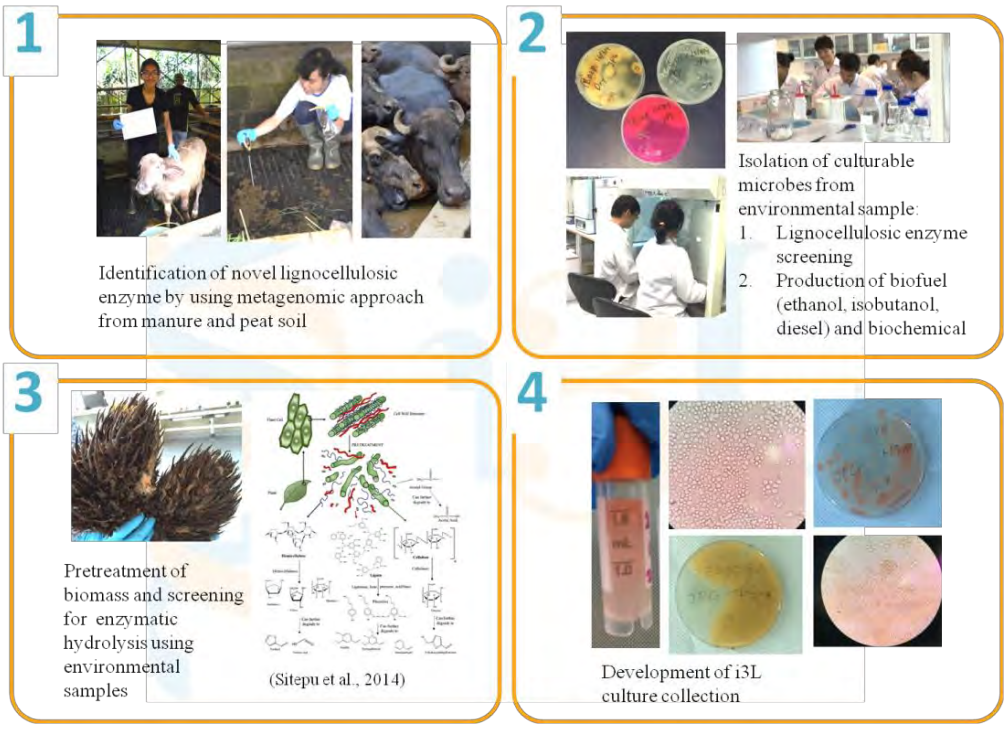

Figure 5: Microbial isolations from environmental samples and EFB pretreatment scenario

Pretreatment and enzymatic hydrolysis of oil palm empty fruit bunch

Hydrolytic efficiency of novel enzymes identified from the environmental samples will be tested on oil palm EFB. Oil palm EFB was obtained from 4-year old oil palm trees planted in Goaboma village, Monterado, district Bengkayang in West Kalimantan. Prior to enzymatic hydrolysis, the EFB will be pretreated with formic acid: water : HCl organosolvent to allow liberation of lignin, cellulose and hemicelluse (Figure 2) (Ferrer *et al*., 2011). The liberated polymers are then subjected to enzymatic reaction (Sitepu *et al*., 2014a), using native and commercial enzyme Novozymes Cellic® CTec2 to degrade the polymer into monomer or dimer molecules that will be fed to microbes for biofuel conversion.

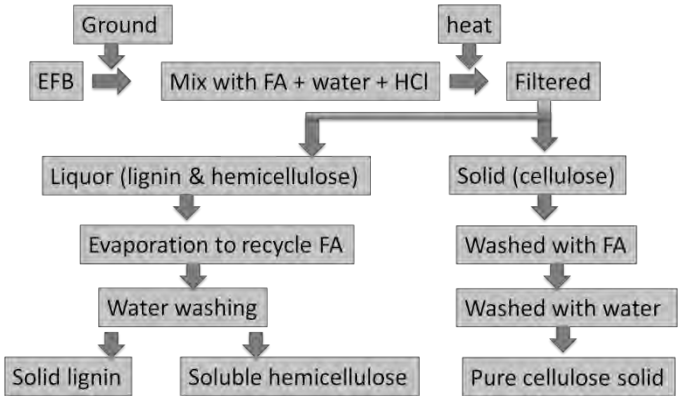

Figure 6: Pretreatment of EFB using organosolvent following methods by Ferrer *et al.* (Ferrer *et al*., 2011)

Bioinformatic and metagenomics

In house database is generated to gather information from available database about known ligninolytic and cellulolitic enzymes and the microbes that synthesize them. A separate local database is prepared for known microbes and enzymes of Indonesia origins that have been studied and published online. Then, the two databases are linked to map out all possible enzymes that may present in these microbes found in Indonesia. Potential microbes are prioritized for screening against lignocelluloses substrate. In line with this activity, metagenomic analysis from manure and peat soil will be conducted to screen for enzymes from environmental samples (Figure 3).

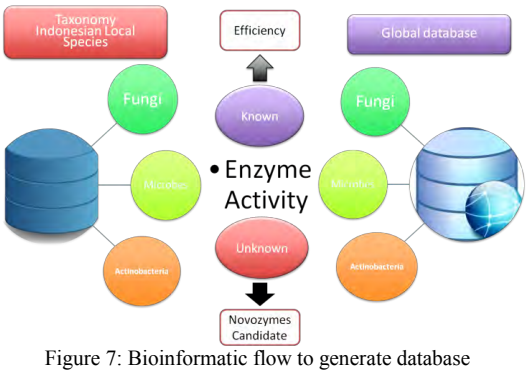

Figure 7: Bioinformatic flow to generate database

Conversion of hydrolysate to fuel

Hydrolysate that is generated from oil palm EFB is fed to microbe for bioconversion to biofuel. In this case, oleaginous yeasts collected from the environmental samples are tested for growth and lipid accumulation in EFB hydrolysate. Candidates that show high growth are scaled up to get enough material to investigate intracellular lipid accumulation following protocols described in Sitepu *et al.* (Sitepu *et al*., 2014b)

RESULTS AND DISCUSSION

This section provides preliminary results of the study. All the activities outlined in the methodology section are still ongoing and more data/results are still gathered.

We selected environmental samples that would likely harbor diverse microbes that can break down lignocelluloses and/or convert biomass to biofuel. The taxon composition of culturable microbes is presented in Figure 4. Isolation of microbes from the environmental sample is ongoing. Colony morphology observation indicated diverse filamentous fungi, yeast and bacteria present in this sample (Figure 5). Identification of the microbe using ribosomal sequencing is ongoing.

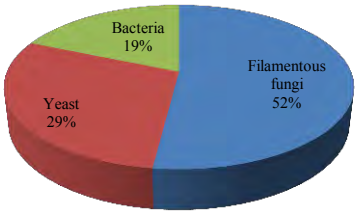

Figure 8: Ratio of filamentous fungi, yeast and fungi isolated from environmental samples

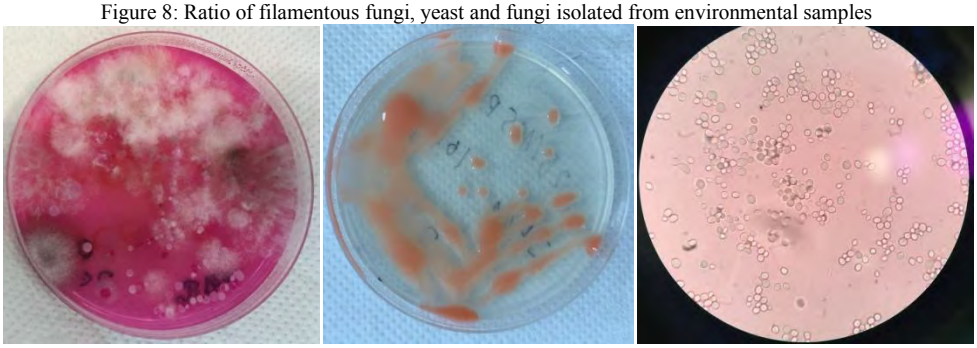

Figure 9: A. Different colonies of microbes grown in RBCA media; B. Yeast grown on PDA; C. Yeast cells under microscope with 1000X magnification

TENTATIVE CONCLUSIONS

The rich biodiversity of Indonesia‘s microbes serve as potential source for accelerating degradation of biomass and conversion of the biomass into valuable chemicals such as biofuel. Our preliminary results indicated that environmental samples collected from buffalo manure harbor diverse microbes and they are currently investigated for their ability to bioconvert oil palm empty fruit bunch to biofuel.

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