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Palm Oil Mill Effluent Metagenome for Cellulose-Degrading Enzymes

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Abstract: Functional metagenomic approach incorporating metagenomic DNA library construction and high-throughput screening has proven to be a powerful tool for identifying novel biocatalysts (Wouters et al. 2014). Culture enrichment strategies are additional pre-screening methods employed to provide an attractive means of enhancing the screening hit rate. In this work metagenomic DNA libraries were generated from Malaysian palm oil mill effluent (POME) microorganisms. Three different samples, namely fresh, ambient-cooled and anaerobic POME microorganisms were inoculated in a medium under controlled temperature, light and pH conditions, and in the presence of carboxymethylcellulose (CMC) for short incubation time in order to allow growth of microorganisms with cellulose-degrading capabilities. Quantitative and qualitative metagenomic DNA tests indicate the presence of high number of microbes in anaerobic POME compared to other samples which guided us to use this particular sample in further experiments. Titer test also showed that the number of enriched-library clones is 5 to 7 times higher than non-enriched anaerobic POME. The use of such a combination of enrichment strategy with metagenomics greatly improves the screening process for biocatalysts.

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INTRODUCTION

The significance of bioprospecting for enzymes lies in their role as promoters of the exponential increase of chemical reactions rates (Anbu et al. 2015). Microorganisms represent two of the three domains of life and contain vast diversity that is the product of an estimated 3.8 billion years of evolution (Riesenfeld et al. 2004). They are considered to be the richest and easiest resource of enzymes, and they are the most physiologically diverse and metabolically versatile (Turnbaugh and Gordon 2008). To fulfill the enzymes requirement with desired characteristics, biotechnological researches focus on this microbial resource due to their easy and high reproduction compared to other higher order organisms such as animals, plants and insects. Microorganisms are also easy to be genetically modified. Enzymes sourced from microbial origins are also reported to function at environmentally friendly conditions with high activity and stability (Uchiyama and Miyazaki 2009; Anbu et al. 2015).

Due to the need for energy security and environmental policies, the world is in need of reliable, safe and clean renewable energy (Misra et al. 2016). The novel trend of green technology by the production of biofuel from agro-wastes gives one the possible solutions. The utilization of these agro-wastes also allow for better management of the wastes by converting them into biofuels (Refaat 2010). In the saccharification stage of biofuel production, efficient enzymes with high catalytic activity are needed (Hill et al. 2006). However, only a handful number of enzymes are currently utilized on the industrial level indicating the need for further research to bioprospect and produce novel specific and efficient enzymes with greater diversity (Prakash et al. 2013).

In this research, POME was chosen as the source for the total habitat microbial DNA. POME, when fresh, is a thick brownish colloidal mixture of water, oil and fine suspended solids and fine cellulosic fruit residues. It is hot (80-90 °C) and possess a very high biochemical oxygen demand (BOD), non-toxic as no chemicals are added to the extraction process (Khalid and Mustafa 1992; Ma et al. 1993), and also acidic with a pH of around 4.5 as it contains organic acids in complex forms that are suitable to be used as carbon sources (MdDin et al. 2006). POME contains cellulosic materials and good source of nitrogen, phosphorus, potassium, magnesium and calcium, which are all vital nutrient elements (MdDin et al. 2006; Habib et al. 1997; Muhrizal et al. 2006) for cellulosic enzymes. This makes POME a favourable habitat for microbes with cellulose-degrading activity.

The inability or difficulty to cultivate microbes in the laboratory conditions has deprived researchers to access the vast majority of these microbes from genomic screening (Riesenfeld et al. 2004; Amann et al. 1995; Jeon et al. 2009). This serious microbiological challenge has been solved with a new approach, known as metagenomic technique, which

can analyse the microbial communities missed by the traditional cultivation and colonies isolation methods. The metagenomic approach is a molecular scan of microorganisms of special habitat permitting the screening of the microorganisms' genetic patrimony without any cultivation and colonies isolation (Simon and Daniel 2010; Mewis et al. 2013). Depending on the screening type, two metagenomic approaches can be distinguished: function-driven or sequence-driven metagenomic. In the present work functional metagenomic approach-a powerful tool in finding biocatalysts including cellulose-degrading enzymes. In this approach no sequence information is required but it has the potential to identify new classes of genes encoding either known or novel function (Kennedy et al. 2011). The construction of functional metagenomic DNA library has the same principle of genomic DNA library construction but the difference is the extraction of the whole microorganism's DNA in this new approach is achieved without sample cultivation and individual microbial isolation.

In metagenomic screening process, the target gene represents a small portion of the DNA sequence. Bioprospecting of target gene can be significantly improved by applying one or several enrichment options (Cowan et al. 2005). Enrichment of samples before metagenomic DNA extraction is an additional pre-screening method to facilitate and enhance enzyme screening by the addition of chemical compounds that serve as substrates to be hydrolyzed by the enzyme in a medium almost similar to the initial habitat-medium. It is an important, but a non-compulsory step to increase the possibility of finding the desired enzymes. It uses the principle of natural selection, wherein a mixed microbial population is inoculated in a medium of defined chemical composition and allowed to grow under controlled conditions (temperature, air supply, light, pH, etc.) in order to favour growth of a particular type of microorganisms with specific characteristics (Kaul and Asano 2012).Cellulose constitutes the major component (35-50%) of plant biomass (Wyman 1994). It is considered as the most available organic substrate and it is known that it is a sustainable source of energy to produce biofuel as potential alternative to fossil-fuel. Cellulose-degrading enzymes are indispensable in the process of degradation of cellulose in plant biomass to obtain fermentable sugars in order to produce biofuels in subsequent processes. However, currently few industrial enzymes are used in this process with less diversity and specificity (Lekh et al. 2014; Sudarshan et al. 2014). This, parallel with the high cost and less efficiency are the main reasons to explore and find natural enzymes from microorganism's consortia, and the preferable method to study these communities is the metagenomic approach. POME is a rich habitat for microorganisms containing nutrient components and growth factors. In the presence of significant quantity of cellulosic fruit residues, some microorganisms can secrete cellulose-degrading enzymes to adapt and survive in POME environment. These conditions make POME as a good medium to bioprospect cellulose-degrading enzymes.

MATERIALS AND METHODS

Sample's collection

The samples of palm oil mill effluent were collected from FELDA Palm Industry Sdn. Bhd. mill near Bentong, Pahang, Malaysia. The POME samples were collected in clean and sterile containers from 3 types of POME; fresh (comes directly from the mill), ambient-cooled (after disposition to air for days) and anaerobic (supplemented with microbes from palm oil mill effluent from the first usage). The pH of samples was 4.2 and temperature of fresh POME was 60 °C and ambient-cooled and anaerobic POME was ≈37 °C. The metagenomic DNA extraction was carried out immediately after sample collection to avoid loss of genetic patrimony.

All chemicals, biochemicals and consumables used in this research were of analytical grade and commercially purchased from Next Gene Scientific Sdn. Bhd, MedigeneSdn. Bhd., Bumi Pharma Sdn. Bhd., and Fisher scientific.

Culture enrichment

Enrichment medium that mimics the composition of POME was prepared with the addition of certain quantity of carboxymethylcellulose (CMC) as substrate to be hydrolyzed by only microbes with cellulose-degrading activity. Sterile acidic minimal media was prepared and adjusted to pH 4.2 (pH of POME). The enrichment medium contained important elements present in POME (Table 1) and 10g/L of CMC, with 1 mL of POME sample. Total volume of 1.2L of inoculated medium was incubated in 6 erlenmeyer flasks at room temperature (25°C) without shaking during 10 days for anaerobic and ambient-cooled samples and the fresh inoculated medium was incubated in 60°C with shaking (200 rpm) for one week.

Metagenomic DNA extraction

The metagenomics DNA was extracted from enriched and non-enriched POME samples using Meta-G-Nome^{TM} DNA isolation kit commercially available from Epicentre Biotechnologies, Madison, WI, USA according to the manufacturer's chemical protocol (Fig.1).

Fosmid cloning

The CopyControl^{TM}fosmid library production kit with pCC1FOS^{TM} fosmid vector (Epicentre Biotechnologies, Madison, WI, USA) was used to construct metagenomic DNA library from POME samples. The cloning procedure has skipped the size selection step because of the advantage of using Meta-G-Nome^{TM} DNA isolation kit which creates DNA sequences of ~40kb. The metagenomics DNA

was end-repaired with end-repair Enzyme Mix to generate blunt-ended 5'-phosphorylated DNA. Metagenomic DNA was purified from 20 cm long 1% low melting point (LMP) agarose gel electrophoresis at 30-35 V overnight. The electrophoresis was performed in the absence of ethidium bromide and UV light; just a portion of the gel was stained and visualized under UV to locate the metagenomics DNA in the non-stained gel. The gel was recovered using GELase enzyme preparation (Epicentre Biotechnologies, Madison, WI, USA). The end-repaired DNA was ligated to pCC1FOS using Fast-Link DNA Ligase, the ligation product was used for phage packaging transformation to *E. coli* EPI300T1 with MaxPlax Lambda Packaging Extracts and plated on large petri-dish with LB agar and 12.5 µg/µl chloramphenicol and incubated overnight at 37°C. All these steps were according the manufacturer's protocol provided by Epicentre Biotechnologies (Fig.1).

Titer test

A titer test has been carried out before the lambda packaging step. At this point, the fosmid clones can be packaged using a small amount of ligation 1 to 2 µl and 200 µl of cells, it allows to test the packaging efficiency as well. The titer test was performed before plating the library to determine the number of plates and phage dilution required to obtain a library that meets our needs. Several dilutions were carried out ($1:10^1$, $1:10^2$, $1:10^3$ and $1:10^4$) to determine the colony forming units per ml (cfu/ml) using the following equation:

$$\text{cfu} = \frac{\text{number of colonies} \times \text{dilution factor} \times 1\ \mu\text{l}}{\text{volume of phage plated } (\mu\text{l})}$$

Table 1. Culture medium of POME enrichment. Sterile acidic minimal medium with pH 4.2.

Composition	Concentration (g/l)
CMC	10
Yeast extract	1
$MgCl_26H_{2O}$	0.6
NH_4NO_3	0.75
KCl	2
K_2HPO_4	1
CH_3COOH	Adjust pH to 4.2

RESULTS

After samples filtration, the filtrate is the source of POME microbes in the metagenomic DNA extraction following cells lysis, the DNA is conserved in TE buffer for the next step. The DNA cleavage and end-repair generate DNA sequences of around 40 kb ready for cloning when we use Meta-G-Nome DNA isolation Kit provided by Epicentre Biotechnologies otherwise an LMP agarose gel electrophoresis can be used to select the DNA with desired size and it can be recovered using LMP agarose GELase enzymes. The recombinant DNA is constructed by the ligation between metagenomic DNA and fosmid vectors pCC1FOS1with ligase enzyme. Packaging system is used to transform the recombinant DNA into EPI300T1R host cell. After plating the transformed *E. coli* on agar large petri dishes with the antibiotic (chloramphenicol), each single colony is inoculated in 384-well microplates with LB medium and 10% glycerol to build the metagenomic DNA libraries.

Metagenomic DNA concentration

In order to test the efficiency of enrichment strategy, we provided a comparison of metagenomic DNA extraction yields for both enriched and non-enriched cultures. The quantity of the observed metagenomic DNA in the microtubes at the end of the experiment shows the important quantity of anaerobic POME and enriched-anaerobic POME DNA compared to other samples. One drop spectrophotometry system was used to test the concentration of different DNA samples by using only 2 µl of samples. The DNA concentration of anaerobic POME is higher than the two other DNA samples. The enriched anaerobic DNA concentration is the highest overall DNA samples (Tables 2 and 3).

LMP-agarose gel electrophoresis

DNA extracted have been analysed quantitatively with LMP-agarose gel electrophoresis (only large gel must be used to visualize DNA sequences > 20 kb and spectrophotometry. It has been used for two reasons, first to analyse DNA fragments size and concentration and to be able to recover the DNA. The electrophoresis results as shown in Fig. 3 the desired sequence size (≈40kb) and the high concentration of enriched-anaerobic (Fig.2).

Fosmid libraries plating

The Petri-dish on the left of figure 4 (A), is the transformed *E. coli* EPI300T1 from non-enriched cultures; the average number of colonies is between 70 and 100 colonies per Petri-dish. Overnight incubation was not enough for some colonies' growth, as such incubation time was extended for a few additional hours which caused the over-growth of some colonies. The Petri-dish on the right of figure 4 (B), is the transformed *E. coli* EPI300T1 from enriched cultures; the average number of colonies is between 400 to 600 colonies per Petri-dish.

DISCUSSION

The current study was initiated to access POME microbes with desired enzymatic activity and to increase the hit rate of enzymes screening with enrichment strategy. In order to enrich for the desired enzymatic activity, microorganisms can be manipulated through medium supplied with chemical compounds that serve as substrates to be hydrolysed by the target enzymes. This experiment is not about microorganisms' cultivation for

enzyme production; it is an enrichment of the culture immediately before the metagenomic DNA extraction to favour the growth of only microorganisms of interest, while microbes without the ability to secrete cellulose-degrading enzymes have difficulties or inability to survive in the presence of high concentration of sugar analogue, CMC. Due to this reason the number of unwanted microbes becomes relatively less compared to microbes with the desired activity. Thus, the increase of the desired microbes' is expected to coincides with the increase of DNA with genes encoding cellulose-degrading enzymes.

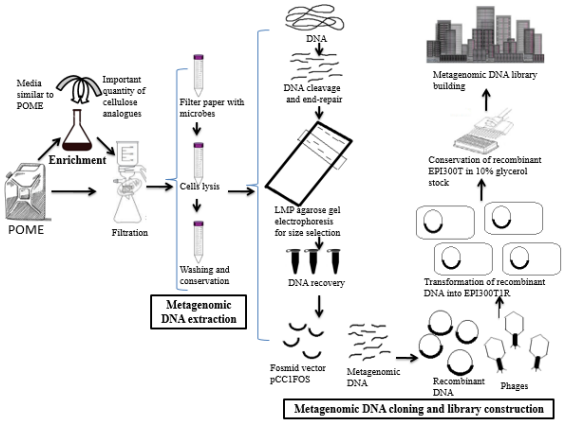

Figure 1. Principle steps of metagenomic DNA libraries construction from POME samples with and without enrichment.

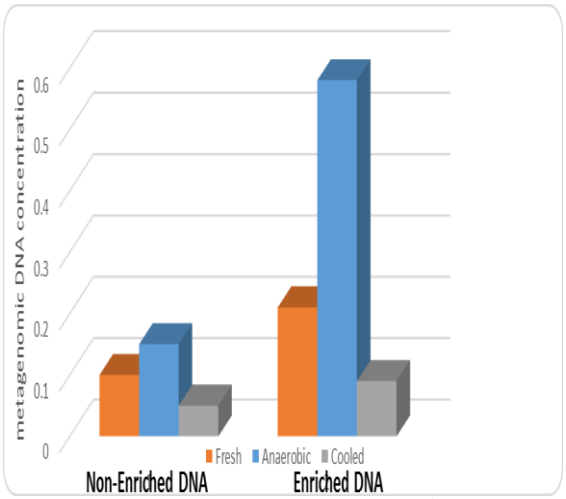

Figure 2. Metagenomic DNA concentration of non-enriched DNA extracted directly after sampling and from enriched samples after exposition step. The concentration of enriched DNA is more significant in all the three types of POME samples. In the enriched anaerobic sample the concentration of DNA is found to be six times more than the non-enriched anaerobic sample.

Medium that mimics the composition of POME was prepared with the addition of certain quantity of carboxymethylcellulose (CMC) as substrate to be hydrolyzed by only microorganisms with cellulose-degradation activity. The CMC in the medium provides the natural selection of our interested microorganisms to enhance the screening in subsequent steps.

Table 2. Types of POME samples and conditions of metagenomic DNA extraction.

Type of POME sample	Volume of sample (liter)	Time to metagenomic DNA extraction (hour)	Type of extraction method	Metagenomic DNA concentration (µg/µl)
Fresh	0.5	3	Chemical/lysozyme	0.1
Anaerobic	0.5	3	Chemical/lysozyme	0.15
Cooled	0.5	3	Chemical/lysozyme	0.05

Table 3. Types of enriched-POME samples and conditions of metagenomic DNA extraction

Types of POME sample	Volume of POME / Volume of medium (ml/ml)	Volume of medium (liter)	Enrich-ment substrate	Conditions (temp., °C / speed, rpm)	Incubation time (days)	Type of extraction method	Meta-genomic DNA concentra-tion (µg/µl)
Enriched Fresh	1 / 200	1.2	CMC	60 / 200	7	Chemical / lysozyme	0.21
Enriched Anaerobic	1 / 200	1.2	CMC	RT / NO	10	Chemical / lysozyme	0.58
Enriched cooled	1 / 200	1.2	CMC	RT / NO	10	Chemical / lysozyme	0.09

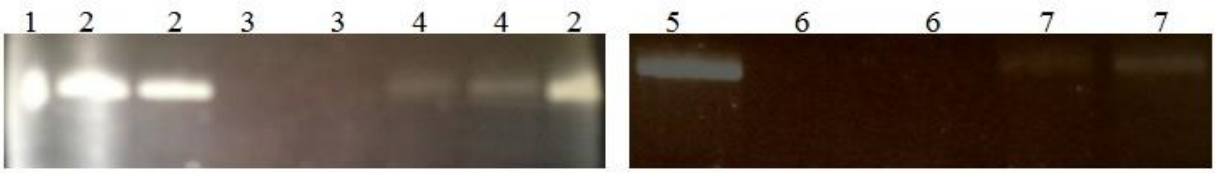

Figure 3. LMP agarose gel electrophoresis of enriched and non-enriched metagenomic DNA of POME. 1: fosmid control (1µL, 100ng of DNA) , 2: enriched-anaerobic, 3: enriched cooled, 4: anaerobic, 5: enriched- fresh, 6: cooled, 7: fresh. The enriched-anaerobic metagenomic DNA showed the highest quantity of DNA; it is 5 to 7 times higher than non-enriched metagenomic DNA, while the quantity of enriched-fresh is 5 times more than non-enriched fresh sample.

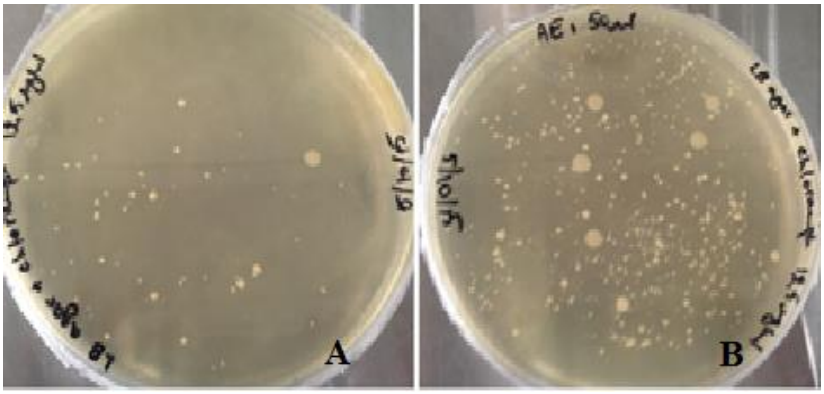

Figure 4. Large Petri-dishes (150 mm × 15 mm) of transformed *E. coli* EPI300T1 plated on LB agar with 12.5 µg/ml chloramphenicol.

Based on the same principle of genomic DNA extraction, metagenomic DNA can be directly extracted by cell lysis within sample matrix "for high yield and low metagenomic DNA size" (Voget et al. 2003) or indirectly when extraction of cells from the environmental material prior to the lytic release of DNA "for low yield and high metagenomic DNA size" (Kauffmann et al. 2004). Meta-G-Nome ^{TM} DNA isolation kit is capable of isolating randomly sheared high molecular weight DNA (≈40 kDa), free of humic acid, directly from homogenized core samples of culturable of difficult-to-culture microbes. This kit is also compatible with the kit used for fosmid cloning in term of recommended insert size (≈40kb).

For the first observation, LMP-agarose gel electrophoresis analysis indicated that there was significant DNA concentration difference of anaerobic POME's metagenomic DNA sample compared to the two other samples, namely fresh and cooled POME. Similar observations were also noted for the enriched-anaerobic POME sample in comparison to enriched and non-enriched fresh and cooled POME (Tables 1 and 2; Fig. 2 and 3).

Molecular cloning is an "*in vivo"* amplification method which permits the study of genetic information and protein expression as well. DNA library is a community of clones resulting from a DNA cloning process. Based on the initial LMP-agarose gel electrophoresis analysis, only anaerobic and enriched-anaerobic metagenomic DNA were further investigated. DNA fosmid libraries of 35 kb to 40 kb insert size from POME anaerobic and enriched-anaerobic metagenomic DNA were constructed. In cloning experiment, *Escherichia coli* EPI300T1 strain was selected in metagenomic DNA library construction as surrogate host of transformed DNA as it is considered as highly efficient competent cell host of recombinant DNA. The pCC1FOS fosmid (Copy Control Cloning System, Epicentre) was chosen as the cloning vector as it combines two major advantages, the high stability of single copy cloning and the high yield afforded by "on-demand" induction of the clone to high copy number. The pCC1FOS fosmid is also characterized by the capability to control the copy number of clones by addition of arabinose to increase DNA yield (10-200 copies per cell). This advantage makes this vector one of the efficient vectors in functional metagenomic approach.

In order to test the cloning technique success and the proximal number of metagenomic DNA colonies, a titer has been carried out before the lambda packaging step. The titer tests showed significant increase in clone number of enriched library compared to non-enriched sample (five to seven times), which alluded to the importance of the enrichment strategy for improving DNA concentration.

CONCLUSION

The work presented here described the importance of microbes' source choice in the functional metagenomic approach to find biocatalysts. The quantitative and qualitative tests applied on three different types of POME have indicated the anaerobic POME as a rich habitat of microbes with potential cellulose-degrading enzyme. To enhance the yield of the metagenomic library screening yield, this study indicates pre-metagenomic DNA extraction step like enrichment method based on natural selection can increase the yield of metagenomic DNA concentration and the size of metagenomic library from five to seven times. The information obtained in this research will open new horizons towards a full understanding of the enrichment's role in increasing the rate of functional metagenomic screening for novel biocatalysts bioprospecting.

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