Improving plastic degradation by increasing the thermostability of a whole cell biocatalyst with LC-cutinase activity

Maelita Ramdani Moeis¹,²* and Muhammad Farhan Maulana²

¹ Fakultas Sains Teknologi, Universitas Muhammadiyah Bandung
² Sekolah Ilmu dan Teknologi Hayati, Institut Teknologi Bandung

*maelita@outlook.co.id

Abstract. Global consumption of polyethylene terephthalate (PET) increases each year, resulting in considerable buildup of plastic waste in the environment. A whole cell biocatalyst (WBC) with LC-cutinase bound to its outer membrane had been constructed to hydrolyze PET (optimum temperature 55°C). The aim of this study was to improve WBC viability at 55°C by inserting Ef-TU gene from sugarcane into WCB cells, with the hope of improving its hydrolytic activity. Escherichia coli BL21(DE3) was co-transformed with two plasmids, the first contained Lpp-OmpA-LC-cutinase fused gene and the second contained Ef-TU gene. Cells transformed with only the first plasmid were used as control. The cells were grown at 37°C and 55°C and viability was analyzed by total plate count. LC-cutinase activity was measured using pNPB as substrate and its capability to hydrolyze PET was observed by scanning electron microscopy. The presence of EF-TU improved WCB viability at 55°C after 90 minutes incubation and LC-cutinase activity remained stable after 72 hours incubation at 55°C. LC-cutinase activity of WCB with EF-TU was consistently higher than without EF-TU. Scanning electron micrograph of PET sheets incubated with WBC cultures with EF-TU showed larger pockets than without EF-TU.

1. Introduction

Plastic is one of the polymers most often used by humans since its commercial development. Plastic resin production in 2012 reached 288 million tons, increasing by 620% since 1975 [1]. The largest percentage of plastics used today is in the packaging industry - an irony because this model was designed to be disposable, but it turned out to be difficult to remove from the environment [1]. One of the most widely consumed polymers is polyethylene, with global production reaching 140 million tons per year [5]. The impact of this plastic waste is of more concern. Its persistence in the ecosystem make plastic waste as one of the top priority problems that must be resolved [2].

From the various plastic degradation methods, namely photodegradation, thermo-oxidative degradation, and biodegradation, the biodegradation process is a process with moderate efficiency but is safe for the environment [3]. The biodegradation process is characterized by the presence of various organisms that could naturally degrade polyethylene plastics such as Ideonella sakaiensis [4], or the discovery of esterase enzymes from leaf compost through a metagenomic approach such as LC-Cutinase [6]. The biodegradation process is expected to continue to increase its economic value, one of which is by eliminating the protein purification stage, namely the whole cell biocatalyst (WCB) approach [7].
Codon optimization was performed on the LC-cutinase gene and the optimum conditions of the enzyme were determined to be 55°C and pH 8.0 [8]. However, there was a problem in the viability of Escherichia coli BL21 (DE3) which was used as WCB at the thermophilic temperature. Thus, the question arose whether an increase in the viability of the WCB LC cutinase at 55°C would improve the degradation activity on the substrate. Therefore, the aim of this study was to determine whether increased viability of WCB-LC cutinase would improve its degradation activity at 55°C.

2. Methodology

2.1. Materials

The bacterial strain used as host cell for the plasmids was E. coli BL21 (DE3). Plasmid pET32bTUF was a pET32b plasmid which had an ampicillin resistance gene and a pBR322 origin of replication that contained a tuf gene from sugarcane (Saccharum officinarum) under the control of T7 promoter [9]. Plasmid pCC1LCC was a pCC1 plasmid from EpicentreTM which had a chloramphenicol resistance gene and an F-factor origin of replication that contained an optimized Lpp-OmpA-LC-cutinase fused gene under the control of a constitutive promoter [8]. The Whole Cell Biocatalyst (WCB) was E. coli BL21 (DE3) transformed with pCC1LCC which contained the LC-cutinase gene.

2.2. Transformation of E. coli BL21 (DE3) with pCC1LCC and pET32bTUF

Competent cells were made using the CCMB method and transformation was performed by heat shock at 42°C for 30 seconds. Transformants were grown on LB agar containing the appropriate antibiotics (100 ppm ampicillin/25 ppm chloramphenicol) for 16 hours.

2.3. Plasmid confirmation

Confirmation of the presence of the two plasmids was carried out by plasmid isolation, restriction digest, and agarose gel electrophoresis. Presto Plasmid Mini Kit [10] was used for plasmid isolation. FastDigest EcoR1 from Thermo Fisher Scientific [11] was used for restriction digest of the plasmids. EcoR1 was chosen because there was only one EcoR1 restriction site in each plasmid (pCC1LCC and pET32bTUF). Electrophoresis of the plasmids was performed on a 1% agarose gel.

2.4. Viability of WCB transformed with pET32bTUF

After 16 hours of activation, 10⁷ cfu of the activated cells were inoculated into 20 mL LB broth with the appropriate antibiotics and 1 mM IPTG was added to induce the expression of tuf gene. The culture was first incubated at 37°C, 250 rpm for 90 minutes, then was transferred into a 55°C shaking incubator, 150 rpm for 90 minutes. The number of viable cells was calculated using the plate count method, where the culture was spread onto LB agar containing the appropriate antibiotics and incubated at 37°C overnight and the number of colonies was observed.

2.5. Measurement of LC-cutinase activity of the WCB

LC-cutinase activity was measured using p-nitrophenyl butyrate (pNBP) as substrate. WCB culture (5 µl) was transferred into one well of a microtiter plate. Then, 95 µl of sterile LB medium, 5 µl of 10 mM pNBP mixed with 5 µl ethanol 95%, and 90 µl Tris buffer pH 8.0 was added. Blanks were also made with only 100 µl of sterile medium, and 100 µl of the same pNBP, Tris and ethanol mixture. The absorbance was immediately measured at a wavelength of 415 nm (t = 0), followed by incubation at 55°C for 15 minutes. After 15 minutes, the absorbance was measured again at 415 nm.

2.6. Qualitative assessment of plastic degradation activity with scanning electron microscopy

Plastic plates from polyethylene terephthalate (PET) water bottles were cut into squares of 1 cm x 1 cm in size. The plastic plates were placed in WCB culture for three days. The plastic plates were then washed with distilled water and ethanol, then dried in an 80°C oven overnight. The plastic plates were then coated with gold (Au) microparticles to facilitate the conduction of electrons, and observed under SEM at 10,000x magnification, 15.0 kV at the SEMP-EDS UPP Chevron ITB Laboratory.
3. Results and Discussion

3.1. Restriction digest of plasmids isolated from transformed cells.

*E. coli* BL21 (DE3) that had been transformed with pCC1LCC became a whole cell biocatalyst (WCB) because plasmid pCC1LCC contained the Lpp-OmpA-LC-cutinase fused gene whose product was located on the cell membrane. Addition of plasmid pET32bTUF resulted in a WCB with *tuf* gene which could be induced by *IPTG*. After transformation with these two plasmids, the presence of the plasmids were confirmed by restriction digest analysis. The plasmids isolated from cells co-transformed with both plasmids and digested with *EcoRI* resulted in two bands (figure 1), which coincided with the size of each plasmid, 9.6 kb and 7.5 kb, size of pCC1LCC and pET32bTUF respectively. This confirmed the presence of both plasmids in the transformants. From here on, cells with pCCILC would be denoted as L and cells with pET32bTUF would be denoted as T.

3.2. Viability of WCB with added *tuf* gene

The concentration of WCB cells with induced *tuf* gene (650-1600 cfu / ml) was significantly higher than WCB cells with no *tuf* gene (120-300 cfu / mL) or with non-induced *tuf* gene (30-100 cfu / mL). Thus, there was a significant increase in the viability of the WCB which expressed the *tuf* gene after exposure to 55°C for 90 minutes (figure 2).

3.3. Stability of WCB LC-cutinase activity after incubation at 55 °C for 90 minutes

The WCB with an induced *tuf* gene had the highest hydrolytic activity towards pNPB (figure 3). The upper interquartile range of WCB with no *tuf* gene was lower than the median of the activity of WCB with induced *tuf* gene, therefore the difference in LC-cutinase activity of these two WCBs were significant.

3.4. LC-cutinase specific activities of the WCBs

The LC-cutinase specific activity of WCB with induced *tuf* gene was significantly lower compared to WCB without *tuf* gene and with uninduced *tuf* gene (figure 4). WCB with induced *tuf* gene had a specific activity of 0.042 units / cfu, whereas WCB without *tuf* gene and with non-induced *tuf* gene had specific activities of 0.23 units/ cfu and 0.35 units / cfu respectively.

Expression of *tuf* gene in bacterial cells had previously been shown to increase the thermostability of the cells [9, 12]. After incubation at 55°C for 90 minutes, the number of surviving cells of WCB with TUF protein increased up to 20 fold compared to WCB without TUF protein. However the increase in LC-cutinase activity was much smaller compared to the increase in the number of surviving cells, which resulted in lower specific activity. This low specific activity could be caused by a lower concentration of LC-cutinase enzyme on the surface of the cells or because the enzymes on the surface of the cells were still active when the cells had died.

3.5. Stability of WCB’s activity during incubation at 37 °C and 55 °C for 72 hours

After 24 hours and 48 hours of incubation at 55°C, WBC with *tuf* gene, whether induced or non-induced, had higher activities than WBC without *tuf* gene at 37°C or 55°C (figure 5). There was no
significant difference in activity for up to 72 hours between WBC with non-induced or induced \textit{tuf} gene. The LC-cutinase activity of WCB with no \textit{tuf} gene at 37°C decreased during the period from zero to 48 hours and then increased in activity at 72 hours. This surge of LC-cutinase activity at 72 hours might be due to cell lysis, which resulted in the release of intracellular enzymes into the medium. Analysis of sub-cellular location of the LC-cutinase enzyme showed the presence of active enzyme in the cytoplasm [13].

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure2.png}
\caption{Viability of WCB with induced \textit{tuf} gene (T + L ind), non-induced \textit{tuf} gene (T + L non) and no \textit{tuf} gene (L) after incubating for 90 minutes at 55°C. The height of the colored squares shows the length of the interquartile range. Horizontal lines are the median. The length of the vertical line shows the maximum and minimum values.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure3.png}
\caption{LC-cutinase activity of WCB with induced \textit{tuf} gene (T + L ind), non-induced \textit{tuf} gene (T + L non) and no \textit{tuf} gene (L) after incubating for 90 minutes at 55°C.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure4.png}
\caption{Specific activities of WCB with induced \textit{tuf} gene (T + L ind), non-induced \textit{tuf} gene (T + L non) and no \textit{tuf} gene (L) after incubating for 90 minutes at 55°C.}
\end{figure}
3.6. Qualitative results of WCB activity on the surface of plastic plate through SEM analysis
The plastic sheets cut out from PET water bottle were incubated for three days at 55°C and pH 8.0. There were differences in surface contours between the control treatment (sterile medium), and treatments with WCB cultures (figure 6). Treatment with a sterile medium showed that the surface was still smooth, with some dust particles accidentally sticking to the surface. Meanwhile, in treatment with culture of WCB without tuf gene there were visible long curved cracks that changed the integrity of the surface of the test plastic plate, so as if it looks like a wrinkled cloth. Whereas, in the treatment with culture of WCB with induced tuf gene, a wider pocket opening was seen and there was a formed flap that was raised upward.

Figure 4. LC-cutinase activity of WCB with induced tuf gene (T + L ind), non-induced tuf gene (T + L non) and no tuf gene (L) after incubating for 72 hours at 55 °C and 37 °C.

Figure 5. Scanning electron microscopy of the surface of plastic plates after incubation for 72 hours at 55°C in sterile medium (left), in culture of WCB with no tuf gene (middle), in culture of WCB with induced tuf gene (right).

4. Conclusion
The WCB LC-cutinase activity was stable during incubation at 55°C for 72 hours. The LC-cutinase activity throughout the incubation of WCB with induced tuf gene was higher than WCB without tuf gene. This resulted in improved degradation of the plastic plates. Thus it could be concluded that improving the WCB viability at the enzyme’s optimum temperature could improve its effectiveness in degrading plastics.
References

[1] PlasticsEurope 2013 *Plastics – the facts 2013* Brussels, Belgium www.plasticseurope.org/Document/plastics-the-facts2013.aspx?FolID=2.

[2] Thompson RC, Moore CJ, vom Saal FS and Swan SH 2009 Plastics, the environment and human health: current consensus and future trends *Philos. T. Roy. Soc. B* **364** 2153

[3] Shah AA, Hasan F, Abdul Hameed A and Ahmed S 2008 Biological degradation of plastics: a comprehensive review *Biotechnol. Adv.*

[4] Tanasupawat S, Takehana T, Yoshida S, Hiraga K and Oda K 2016 Ideonella sakaiensis sp. nov., isolated from a microbial consortium that degrades poly(ethylene terephthalate) *Int.J. Sys.Evol. Micr.* **66** 2813

[5] Sivan A. 2011 New perspectives in plastic biodegradation *Curr Opin Biotech*

[6] Sulaiman S, You DJ, Kanaya E, Koga Y and Kanaya S 2014. “Crystal Structure and Thermodynamic and Kinetic Stability of Metagenome-Derived LC-Cutinase” *Biochemistry* **53** 1858

[7] Baixue L and Tao Y 2017 Whole-cell biocatalyst by design *Microb. Cell Fact.* **16** 106

[8] Victorio Y 2017 Optimasi dan karakterisasi whole-cell biocatalyst protein fusi LCC-OMPA-LC-cutinase dan mutan sinonimnya pada Escherichia coli EPI300TM pendegradasi polietilen tereftalat. *Repositori SITH ITB*

[9] Amanda D 2017 Isolation and characterization of *tuf* gene encoding chloroplast elongation factor Tu (EF - Tu) protein from sugarcane (Saccharum officinarum). *Repositori SITH ITB*

[10] GeneAid 2019 Presto™ Mini Plasmid Kit (PDH100, PDH300) | Geneaid Accessed January 27, 2019. http://www.geneaid.com/products/plasmid-dna-purification/plasmidkit-miniprep-presto.

[11] Thermofisher Scientific 2019 FastDigest EcoRI - Thermo Fisher Scientific Accessed January 27, 2019. https://www.thermofisher.com/order/catalog/product/FD0274.

[12] Moriarty T, West R, Small G, Rao D and Ristic Z 2002 Heterologous expression of maize chloroplast protein synthesis elongation factor (EF-Tu) enhances Escherichia coli viability under heat stress. *Plant Science*, **163** 1075

[13] Moeis MR 2016 Novel strategy for degrading PET using whole cell biocatalyst: OMPA-LC cutinase, Grant Ceremony & Seminar on Research Findings assisted by the Asahi Glass Foundation