COMPARISON ON BIODEGRADATION OF LOW-DENSITY POLYETHYLENE (LDPE) MIXED WITH CORN STARCH BY *A. niger, R. oryzae* AND THEIR BIOFILM

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Abstract

This research focusses on the degradation of Low-density polyethylene (LDPE) mixed with corn starch by *A. niger, R. oryzae* and their biofilm. The biofilm was formed using 96 well plates before being observed using Scanning Electron Microscopy (SEM). The mycelial mass was observed in the SEM image of the biofilm indicating the present of both species. The rate of degradation of *A. niger, R. oryzae* and their biofilm were evaluated based on weight loss within 7 days of incubation time and further analyzed using Fourier Transform Infrared Spectroscopy (FTIR). Based on the comparison, the biofilm of *A. niger* and *R. oryzae* has shown higher degradation compared to the fungi alone (without the biofilm), up to 1.87%. In addition, amylase and cellulase enzymes were secreted by the biofilm to accelerate the degradation. Through the FTIR analysis, it was found that there are differences in the polymer structure after 7 weeks of fermentation compared to LDPE control. The bond formation differences before and after fermentation were observed with the changing in carbonyl and ester structures suggesting enzymes secreted by the biofilm had possibly changed the original polymeric structure of the LDPE. Thus, it can be concluded that the biofilm of *A.niger* and *R.oryzae* could be a breakthrough in providing a solution in bioplastic degradation.

Keyword: *A. niger, R. oryzae*, biofilm, degradation, LDPE with corn starch

Introduction

Demand on plastics have increased up to 150 folds, with the production of 1.5 million tons in 1950 and 245 million tons in 2006 (Russell et al., 2011). However, improper management of plastics waste has led to serious environmental and health issues. This include the abundance of plastics waste in land and ocean. Report shows the amount of plastics waste has been doubled over the last 50 years and threaten the survival of many species of wildlife (Geyer et al., 2017).

Thus, an idea occurred to scientists to create a bio-based plastic by combining the synthetic polymer with natural polymer as a substitution. This bioplastic is evolving in market and has a wide range in application because it maintains the characteristics of synthetic plastics but are more environmentally friendly at the same time (Spierling et al., 2018).

Compared to the other types of polymer, Low-density Polyethylene (LDPE) is preferable to be used in bioplastics because it has lower density, is softer and also more flexible compared

to high-density polyethylene (HDPE). Yet, the bioplastics of LDPE mixed with corn starch is more well-known because the combination is cost effective, has good thermal stability, has minimum intrusion with flow properties, and low disturbance of product quality (Sabetzadeh et al., 2012). In addition, the rate of degradation could be enhanced as the starch could reduce the chemical interaction between corn starch and LDPE (Oromiehie, 2012; Hoque, 2013).
Through microbiological approach, *Aspergillus niger (A. niger)* and *Rhizopus oryzae* (*R. oryzae)* had showed their capability in degrading polymer by secreting hydrolytic and oxidative enzymes. In fact, these microorganisms could utilize LDPE as their carbon source efficiently (Awasthi et al., 2017). Biofilm is the communities of microorganisms from same or difference species that can increase the physical support of the microorganism by assembling themselves on surface-associated microbial cells in extracellular matrix such as polysaccharides. Biofilm from plastic-degrading soil may contribute in better degradation (Morohoshi et al., 2018). However, current study may be limited to the comparison between species in the LDPE degradation between species. Thus, in this study, the rate of degradation between biofilm and the fungal alone was compared.

Materials and Methods

Sample preparation

LDPE mixed with corn starch was obtained from Polymer Unit, Universiti Teknologi MARA Perlis. *A. niger* and *R. oryzae* was obtained from Biology Lab, Universiti Teknologi MARA Perlis.

Biofilm Preparation

A. niger and *R. oryzae* were grown on Potato Dextrose Agar (PDA) for 7 days and harvested using 5 ml of Tween 80 (Ranjan, 2019). The suspension containing spores and mycelium were standardized to 10^4 spores/mL. For the kinetic biofilm, 100 μL of spore suspension, 100 μL of culture broth media and 100 μL of sterile tap water were added per well into 96-well at the bottom of polystyrene microtiter plates. Media-only blanks were also set up. The plates were incubated at 30 °C for 24 hours (Lima & Sequeira, 2013).

Culture of Microorganism

Culture Fungi Preparation

Mycelium or spores of *A. niger* and *R. oryzae* were cultured on Potato Dextrose Agar by using a spreader (Freitas et al., 2013). The plates were incubated at 37 °C for 7 days. The developed fungal mats were sub-cultured on Potato Dextrose Agar to obtain pure culture and preserved in slant at 4 °C (Das & Kumar, 2015).

Enzymatic Tests

25 g dumping soil was mixed with 100 mL distilled water and shaken at 160 rpm for 60 minutes at room temperature. The cell-free supernatant was used for the enzyme activity assay (Costa et al., 2017).

Amylase Test

3 g of starch powder were mixed with 100 mL distilled water in 250 ml Erlenmeyer flask and autoclaved at 121 °C for 15 minutes. The solidified media were streaked with *A. niger* and *R. oryzae* and incubated inversely at 30 °C for 48 hours (because the starch hydrolysis begins after 4 hours of incubation). The colonies formed were flooded with Lugol solution for 1 minute before the absorbance was recorded at 620 nm using a spectrophotometer (Freitas et al., 2013). The amylase activity of a sample may be determined by the following equation:

$$\%\ Degradation = \frac{Initial\ weight - Final\ weight}{Initial\ weight} \times 100$$

Cellulase test

Carboxymethylcellulose agar (CMCA) was used to identify the presence of cellulolytic activity in the biofilm by staining or precipitation of undigested CMC in the plate. The clear hollow indicates the presence of the enzyme. All plates were incubated at 27 °C for 16 hours before being measured at 575 nm (Johnsen et al., 2014).

Degradation test on Low-density Polyethylene Plastic Degradation (LDPE) mixed with Corn Starch

Three separated round containers were used for each fungal labelled for *A. niger*, *R. oryzae* and their biofilm. 0.26 g/mL of dumping soil were transferred into the container containing 50 mL Malt Extract Broth (MEB). The flasks were incubated and shaken at 160 rpm for 24 hours to ensure asepsis. A 10^4 serial dilution of spore and mycelium were inoculated in the culture medium and incubated at 30 °C for 7 weeks. Control samples consist of soil and LDPE plastic were also stored for positive and negative control.

The LDPE plastics were cut into 2 cm x 2 cm size. A total of 7 pieces of LDPE were taken using forceps and washed by distilled water and sprayed with 70% alcohol, dried at room temperature, and weighted after 7 days for 7 weeks. The percentage of LDPE degradation was calculated as below:

$$\%\ Degradation = \frac{Initial\ weight - Final\ weight}{Initial\ weight} \times 100$$

Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) was used to identify before and after LDPE degradation structural changes. It is very sensitive to local molecular environment and has been widely applied to investigate the reaction during LDPE degradation between macromolecules (Gajendiran 2016). The FTIR spectra of LDPE plastic films were obtained using a Nicolet™ iS50 FTIR Spectrometer in the range of 4000 – 400 cm^{-1}.

Result and Discussion

Formation of Biofilm

Biofilm of *A. niger* and *R. oryzae* had been observed using Scanning Electron Microscope (SEM) as in Figure 1 at 100X magnification.

Based on **Figure 1**, there are differences in the morphology of *A. niger, R, oryzae* and their biofilm. At 100x magnification, the biofilm shows the combination of mycelia and spores whereas in single cells of the microbes, the morphology was not apparent. In addition, it is expected that the fungal growth in biofilm could be inhibited due to higher competition for space and nutrients, compared to the single cells (Lima & Siqueira, 2013).

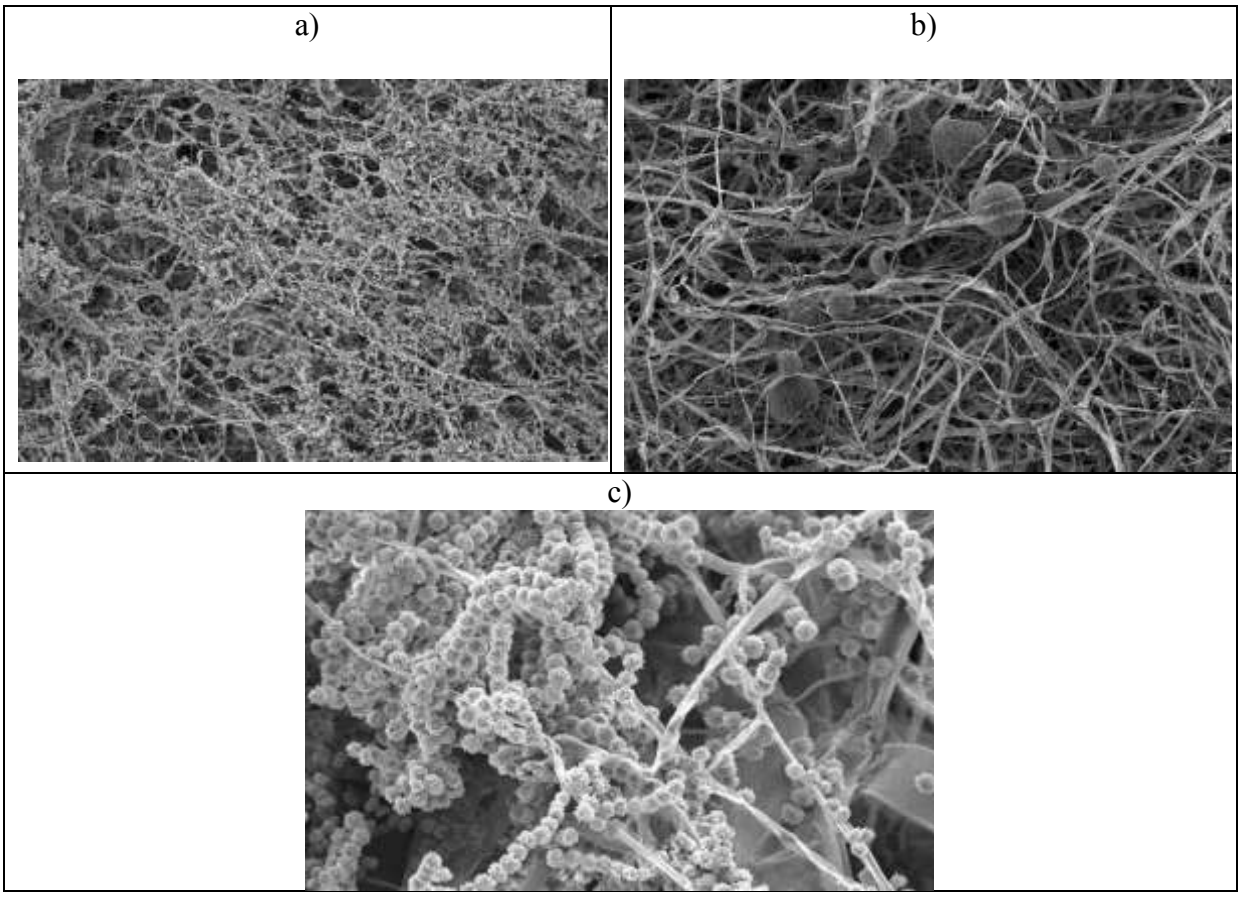

Figure 1 SEM images of a) *A. niger* 100x magnification b) *R. oryzae* 100x magnification c) *A. niger* and *R. oryzae* combine 100x magnification.

Analysis of Weight Loss of LDPE Mixed Corn Starch

The rate of degradation was determined by observing the percentage of LPDE mixed corn starch and LDPE control fermentation weight loss for 7 weeks. The percentage weight loss of LDPE mixed corn starch and LDPE (control) was plotted as in **Figure 2**.

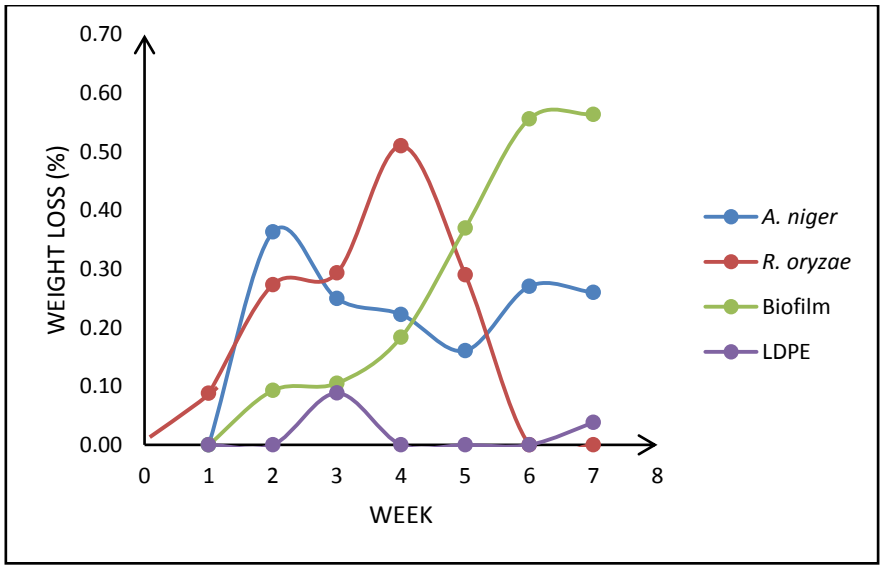

Figure 2 The percentage of weight loss of LDPE mixed with corn starch and LDPE (control)

At the beginning (in week 1 and 2), the highest degradation rate was shown by *A. niger* and *R. oryzae* alone compared to their biofilm. However, the rate of degradation of the fungi alone decreased from week 3 onwards. In contrast, the rate of degradation by the biofilm increased steadily from week 1 to 7. Thus, it can be concluded that the degradation rate by biofilm is higher than the single species alone. This is supported by earlier study, whereby similar result was obtained due to the secretion of different types of enzymes in the mixed culture and the increasing of the enzymatic activities compared to the single culture (Brink et al., 2011). Both mycelium and hyphae worked together to degrade the LDPE (Khan et al., 2017). Additionally, the degradation rate of LDPE mixed with corn starch (negative control) was the slowest.

The weight loss of the LDPE mixed with corn starch by *A. niger, R. oryzae* and their biofilm might be caused by the degradation enzymes secreted by both fungi and the biomaterial from the film itself. The corn starch in the film had enhanced the degradation kinetics and thus increased weight loss (Hoque et al., 2013). This could be due to the hydrophilic nature of starch that can retain the moisture which contributed to the degradation of the polymer. The higher the starch content in the polymer, the higher the moisture content that induced faster degradation. The results show that the incorporation of hydrophilic starch into hydrophobic LDPE enhanced the hydrophilicity and degradability of the overall polymer (Hoque et al., 2013).

Enzymatic Test

Amylase and Cellulase Test

The specific acitivities of amylolytic and cellulolytic enzymes by mixture of *A. niger* and *R. oryzae* biofilm were quantified after being grown on starch agar for 48 hours at 30 °C. **Figure 3** shows the comparison on both enzymes secreted by the biofilm degradation on LDPE mixed with corn starch.

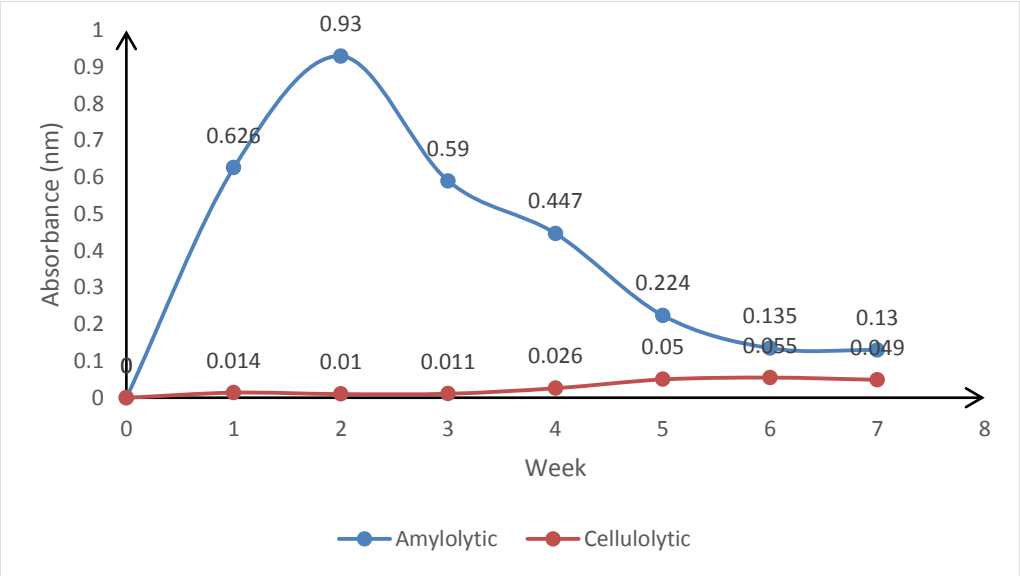

Figure 3 The comparison on both enzymes secreted by the biofilm degradation on LDPE mixed with corn starch

At the early fermentation, higher amount of amylase (0.626 nm) was secreted compared to cellulase (0.014 nm). However, the rate of amylase secretion had decreased from week 3 onwards. In contrast, from week 3 to 7, a higher secretion of the enzyme was shown by

cellulase.

It is probable that during the first stage, the degradation of the LDPE mixed with corn starch happened through the secretion of the amylase. These enzymes could hydrolyze the internal α-1.4-glycosidic linkages in the corn starch and convert them into low-molecular-weight products such as glucose, maltose and maltotriose unit (Mikawlrawng, 2016). At this rate, amylase had reduced the amount of the corn starch in the LDPE which contributed to the weight loss of the plastic through starch hydrolysis. Here, the olygosaccharides from the corn starch might be hydrolysed to simple molecules such as maltose and glucose (Freitas et al., 2013). In contrast, the decreasing rate of amylase from week 3 onwards might be attributed to the temperature and absence or lessen amount of substrate in the medium (Santos et al., 2016).

Instead of starch polymer, the corn starch is built up from cellulose. Since the stucture of cellulose is stronger compared to starch, it results in a delay or slower cellulolytic secretion compared to the amylase. This cellulase also participated in the substrate binding, multi-enzymes complex formation and possibly attached itself to the cell surface (Be´guin and Aubert., 1994). Moreover, carbon in the bioplastics itself plays a role as the source of growth of the fungi before it became depleted and led to the decline rate of the secretion. As a byproducts of this, these polymers will be degraded into CO_2 and H_2O (Ahmed et al., 2018).

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Polymer degradation was reflected in changes of bond scission, chemical transformation and formation of new functional groups. **Figure 4** and **5** show the FTIR of LDPE control and LDPE mixed corn starch ferment in the *A. niger, R. oryzae* and the mixture of *A. niger* and *R. oryzae.*

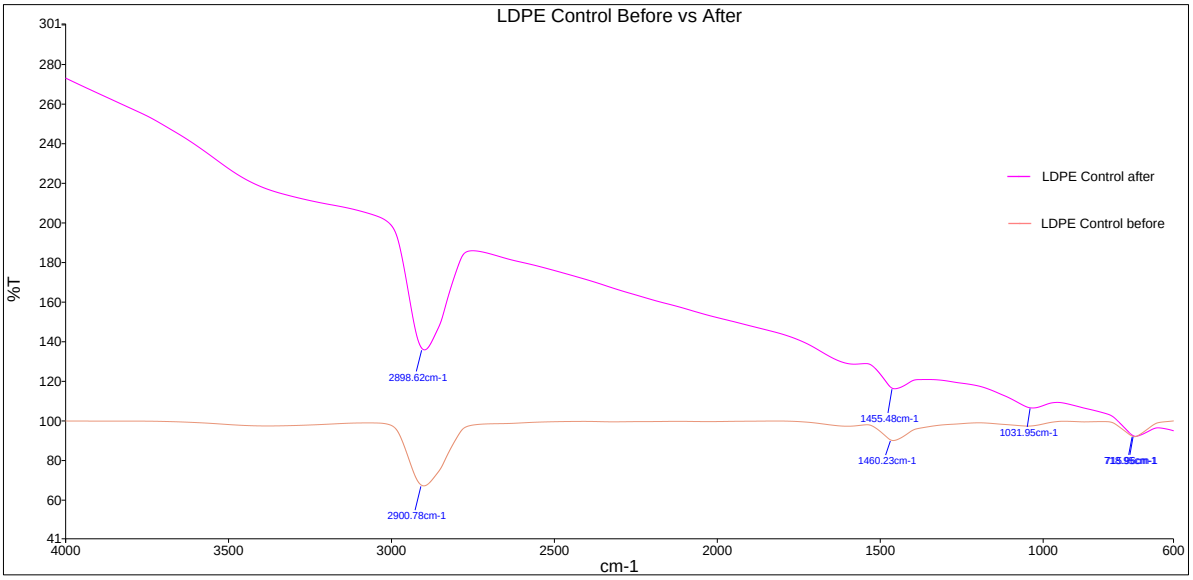

Figure 4 FTIR of LDPE (negative control) Before and After Fermentation

Figure 5 shows the peak observed in LDPE mixed corn starch ferment in *A. niger, R. oryzae* alone and the mixture of *A. niger* and *R.oryzae.*

Figure 5 Comparison of FTIR LDPE Mixed Corn Starch in *A. niger, R. oryzae* and Their Biofilm Flask Before and After Fermentation

Based On **Figure 4** and **5**, there are several changes of FTIR on the surfaces of the bioplastic before and after 7 weeks of incubation. The peak number shows the complex nature of the LDPE with the presence of absorption bands at 736 cm (C-H bend), 1474 cm (C=C stretch) and 2914 cm (C-H stretch). However, there was a modification on the test samples band intensities in different regions after 7 weeks of fermentation (**Figure 5**).

In comparing the before and after of LDPE mixed with corn starch samples in *A. niger* alone, peak differences occurred at 725 cm (C-H bend) and 1027 cm (strong C-O stretch). While in *R. oryzae* alone, the before and after samples did not show any interesting peak. Meanwhile, in the biofilm before and after the incubation, there was an increasing band in saturated –C-C-C stretch attached to carbonyl absorption at 1108 cm, 2553 cm (S-H stretch) and 3285 cm (O-H stretch). The peak observed was remarkably different from the other samples. This is attributed to the formation of carbonyl bond through oxidation of the polyethylene moieties during incubation period. The change in the peak values of almost all functional groups confirmed the configurational change on polymer surface (Awasthi et al., 2017).

Overall, the appearance of new peak can become an indicator for plastic degradation where the decrease in the carbonyl and ester occurred. This phenomenon thus indicates that there was a rupture occurring in the polymeric structure (Khan et al., 2017). Surprisingly, the before and after of LDPE mixed corn starch in *R. oryzae* fermentation shows no differences in the peak appearance. The peak appeared only at 3285 cm (O-H stretch). This shows that there were no changes in the polymeric structure of the LDPE. In *A. niger* fermentation, the FTIR before incubation shows flat result while after fermentation, 725 cm, 1000 cm and 3285 cm peaks were observed. This shows that the original polymeric structure has been changed by the enzymatic activity of the microorganisms.

Conclusion

In conclusion, the biofilm of *A.niger* and *R.oryzae* had successfully been formed. Results show the potential of the biofilm as bioplastic degrader compared to when single species were used. Enzymes such as cellulase and amylase are secreted for the degradation of the LDPE. Both enzymes might 'take turn' in degrading the polymer. The change in FTIR might also be attributed to the enzymatic activities.

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Conflict of interests

There are no conflicts of interest in this research.

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