Identification of Thermoplastic Starch Degrading Species Isolated From Organic Food Waste Compost

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***Abstract*— Thermoplastic starch is a potential alternative in solving the problem of ecological impact from the synthetic polymer. Thermoplastic starch is made of renewable sources and biodegradable. The purpose of this research is to isolate fungi that are able to degrade thermoplastic starch (TPS) from *Tacca leontopetaloides* and observed the fungi growth including enzyme production. In this research study, fungi species was isolated from organic food waste compost. A fungal was selected to further the study. The growth of the fungal was observed on TPS solid medium and thermoplastic starch with acetic acid (TPSAA) solid medium. Lugol's Iodine (IKI) test was conducted to determine its capability to produce starch degrading enzyme. The weight loss was also recorded thought the study. From the research, a fungal is selected as the microorganisms for further study about degradation. According to the morphology and characteristics similarity, the fungal was identified as *Aspergillus sp.* It is found that the *Aspergillus sp.* able to grow on TPS but not on TPSAA due to the acidity of the solid medium. Besides that, Aspergillus sp. was proven as an α-amylase producer when a clearzone was appeared on the media during IKI test. The study found that the weight loss of thermoplastic starch degraded by *Aspergillus sp.* for 2 months is 1.81% .**

***Keywords*— *Aspergillus sp,* fungi, *Tacca leontopetaloides*, thermoplastic starch, enzymne, growth.**

I. INTRODUCTION

The study of the thermoplastic starch is another alternative to pay attention to and starch has become a concern as thermoplastic starch has lower impact on ecological problem. Therefore, a lot of research studies on the degradation of thermoplastic starch (TPS) whether in water, sludge, compost or soil. Several methods have been conducted to test the biodegradability of the TPS in the defined environment.

There are three polymer degrading techniques: energetic, chemical and biological. Energetic degrading technique may consist of thermal or radiant energy such as high-energy radiation like gamma rays, ion beams, and electrons or even low energy radiation like ultra-violet (UV) rays (Ramani & Ranganathaiah, 2000). While chemical degradation is caused by some chemicals like acid or alkalis (Premraj & Doble, 2005). Using certain types or microorganism to degrade polymers is classified as biodegradation method (Premraj & Doble, 2005). When plastics are exposed microorganisms at its suitable growth condition, the biodegradation process will begin. There are a lot of growth conditions that one should consider such pH, temperature, moisture content, oxygen and nutrients.

Some types of plastics have been shown to be either bio-based, biodegradable or both kinds of polymer. For TPS, that is bio based biodegradable polymer, its rheology and mechanical properties are influenced by the plasticizer in obtaining the thermoplastic characteristics (Imre & Pukánszky, 2013). This is because the starch solely is brittle because of its hydrophilic nature, therefore it needs to undergo the melt processing with gelatinization to destroy the crystalline structure of native starch (double helix formation of linear regions of amylopectin) with glycerol as plasticizers.

Fungi are one of the organisms that are responsible for the decomposition of carbon in the biosphere as they are equipped with extracellular multienzyme complexes to metabolize the organic matter (Del Moral, 1974)and break down the natural polymeric compounds into nutrients. In the subkingdom of Dikarya, there are two clades, consist of Ascomycota and Basidiomycota. Through some of the studies, it was found that Ascomycota and Basidiomycota are with the highest potential fungal for plastic degradation (Sasikala and Ramana 1996) such that among the species is *Aspergillus.*

In the progress towards a plastic free world, some of researchers looking in plants that produce starch, such that it does not disturb the corps or food. One of potential plants is *Tacca Leontopeloides*. *T. leontopetaloides* is also one of the renewable plant resources that are capable to produce thermoplastic starch. *T. leontopetaloides* is known as

Polynesian arrowroot starch; it is a wild perennial herb family of Dioscoreaceae . This plant is naturally distributed from Western Africa through Southern Asia to Northern Australia. The advantages of using starch are cheap, abundant and renewable.

II. METHODOLOGY

A. Preparation of thermoplastic starch (TPS) solid medium

The *T. Leontopetaloides* starch powder was obtained from Mersing, Johor. The starch powder is the main medium for making the TPS solid medium. Two types of TPS slid medium were prepared, namely TPS with acetic acid (TPSAA) and TPS only. Both agar were prepared with different compositions but at the same gelatinized temperature (85-90°C). TPS solid medium consists of distilled water, glycerol and *T. leontopetaloides* starch while another TPS solid medium has an additional of 5% acetic acid. The ratio for the TPS agar is 9:1:2 while for TPSAA is 8:1:2:1 respectively. The prepared solution was then autoclaved and poured into the sterile petri dish and sealed with parafilm. Along the way an aseptic technique was applied. The petri dishes were kept in a fridge before it could be used.

B. Preparation of Potato Dextrose Agar (PDA)

1L of PDA agar was prepared by dissolving 0.39g of PDA powder with 1L of distillation water at 121°C. Autoclaves PDA agar solution was then poured on sterile petri dishes and sealed with parafilm twice.

C. Source of compost soil

Compost soil sample was obtained from Pasar Seksyen 6 Shah Alam. The compost soil was made from wastes of vegetables, fish and fruits that were converted into soil using composting machine by Mentari Alam EKO (M) Sdn Bhd.

D. Inoculation of fungi species

10g of compost soil with 80ml of distilled water were placed into the Falcon tube and mixed using a vortex mixer. The suspension was then transferred into the TPS solid medium petri dish to observe any growth of microorganism. Any microorganism that grew on the TPS solid medium was then transferred to PDA agar for isolation of only fungi species.

E. Identification of Aspergillus sp.

The fungi species obtained with different physical characteristic were isolated to individual PDA petri dishes until only pure culture was obtained. *Aspergillus sp*. was identified according to its physical morphology and observed under light microscope. Starch hydrolysis test using Lugol's solution also was conducted to detect amylase enzyme production.

F. Observation of Aspergillus sp. Growth

The growth of Aspergillus sp. was observed for one month on the TPS and TPSAA solid medium.

G. Preparation of TPS films

The TPS films were prepared with distilled water, glycerol, *T. leontopetaloides* starch and 5% acetic acid with a ratio 8:1:2:1. The 250ml mixture was stirred gently on the hot plate using a glass rod until it was homogenized. The homogenized mixture was poured onto a tray and left to dry until constant weight is achieved. The film was peeled from the tray and roll-milled for constant surface and thickness.

H. Weight lost measurement

TPS plastic strips were cut into 2x2cm and were dipped into the *Aspergillus sp.* suspension and placed on the sterile Petri dish and incubated at 37°C. The initial (0 day) weight and the weight loss were recorded for two months until the observation was complete.

III. RESULT & DISCUSSION

A. Identification of Aspergillus sp.

Several species of fungal belonging to Ascomycete, Basidiomycete and Deuteromycete groups were isolated from the composted soil in Pasar Shah Alam. Then, one fungal species is isolated for further study purposes and a pure culture of interested fungi was isolated. According to Geweely & Ouf, 2011, the highest efficient fungal degraded starch based starch polymers is *Aspergillus Niger*. Therefore a pre-judgment was made and a culture with black spore was selected.

Fig. 1: Pure culture of *Aspergillus sp*. Under the naked eye

Throughout the observation as Figure 1 shows, presents the characteristic of the fungi species isolated from the compost soil observed under naked eye. It had shown the characteristic of powdery spore in black colour. The entire

colony looked 'pixelated', made up of millions of tiny spots like one of Seurat's pointing paintings.

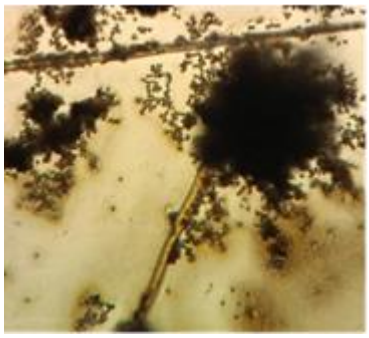

Fig. 2: *Aspergillus sp*. under light microscope (40x)

Figure 2 presents the characteristic of the fungi species isolated from the compost soil observed under light microscope. The individual fungus looks like dandelions, with a transparent yellowish stem that supports the dark brown headshot which radiates out from the centre with many spherical spores.

Throughout the observation, it can be stated that the fungal species is *Apergillus sp*. The strain belongs to the genus *Apergillus* Section *Nigri*. The characteristics are similar. Accordining to Silva et.al., 2011, Aspergillus Section Nigri. characteristically presents dark-brown to black conidia, with conidiophores, spherical vesicles and light pigmented hyphae vesicles and lightly pigmented hyphae near the apex(Silva et al., 2011)

Kingdom	**Fungi**	**Order**	**Eurotiales**
Phylum	Ascomycota	**Family**	Trichomaceae
Class	**Eurotiomycetes**	**Genus**	***Aspergillus***

Table 1: Taxonomy of *Aspergillus sp.*

Even though it can be classified under section Nigri but the result cannot make an affirmation of the species as the *Aspergillus* species are morphologically very similar to each other and makes it difficult to distinguish them based only on morphological information.

Nevertheless, *Aspergillus sp*. is an asexual spore-forming structure that posesses the ability to grow at a high osmotic concentration.Usually, it can be found at oxygen-rich environments in the form of molds, and contaminates food with starch based.(Ibrahim, Maraqa, Hameed, Saadoun, & Maswadeh, 2011)

B. Observation of Apergillus sp. growth

Biodegradation is the decomposition by the action of the enzyme secreted by microorganisms. There are three methods of study of biodegradation filed (in vivo), simulation and laboratory (in vitro). In vitro methodology was use for this study due the degradation rate of individual fungus could be determined clearly(Kale, Deshmukh, Dudhare, & Patil, 2015).

Fig. 3: Growth of *Apergillus sp.* on TPS solid medium (1 week)

Fig. 4: Growth of *Apergillus sp.* on TPS solid medium (2 week)

Figure 3 shows the growth of *Aspergillus sp.* on the first week while Figure 4 shows the growth of *Aspergillus sp*. on the second week. *Aspergillus sp*. is a fast growing fungus; on day two, the solid medium was already peppered with black dots, and several yellow spots afterwards. The colony changed to black after a few days, eventually coalesce to form a black mass. From the naked eye observation on the third week, on the top of medium agarseveral patches of black powdery mass were formed. At week 4 onwards, the plate was watery. The situation occurred because water droplets produced from *Aspergilllus sp.*'s respiration did not have exit space as the petri dish was sealed. Therefore, no significant growth can be seen through the observation and the plate was flooded.

Figure 5 and Figure 6 showed the growth of *Aspergillus sp.* on TPSAA solid medium. Throughout the observation until 2 months there was no difference and not a single colony of Aspergillus sp. had grown. According to (Higgins & Brinkhaus, 1999) the growth and morphology of fungi are influenced by the pH media.

Fig. 5: Growth of *Apergillus sp.* on TPSAA solid medium (1 week)

Fig. 6: Growth of *Apergillus sp.* on TPSAA solid medium (2 month)

Therefore the reason for having no growth of *Aspergillus sp*. on TPSAA was due to the addition of acetic acid, resulting the agar pH value to decrease. With low pH value, the media

is in an acidic state thus it influenced the growth of *Aspergillus sp*. by acidifying the cell. It consumed a great amount of energy to maintain the intracellular pH homeostasis(Kang, Park, & Go, 2003). Therefore, by lowering media's pH, it will cause inhibition of microbial growth and as *Apergillus sp.* is vulnerable to the presence of organic acids (acetic acid), it will cause antimicrobial effects. This phenomenon is due to the hydrophobic feature of most of organic acids, which allows free diffusion of the protonized form through the cell membrane. This diffusion process takes place spontaneously due to pH and osmolarity gradients that exist between the inner and outer sides of the cell (Hassan, El-Kadi, & Sand, 2015). The decrease of intracellular pH by releasing the proton causes intracellular pH to be higher than the extracellular. The acid undergoes dissociation as soon as it enters the cytoplasm. In order to counter the decrease of cytoplasmic pH resulting from the ionization of entered acid, the cell allocates the main part of its energy content to eliminate these newly formed protons which results in slower growth kinetics (Peláez et al., 2012) or even to no growth on the media.

C. Production of enzyme

Most screening methods devised for the detection of amylolytic microorganisms involve growing them on solid media containing soluble starch and testing for starch hydrolysis by flooding the plates with iodine solution (BALKAN, AYDOĞDU, BALKAN, & ERTAN, 2012). The use of plate culture method containing starch as a carbon source is a simple and rapid way to screen amylolytic microorganisms because production of amylase is highly dependent on starch.

Fig. 7(a)

Fig. 7(b)

Fig. 7(c)

Fig.7 Starch hydrolysis test (a) before, (b) after –top (c)after-bottom

Figure 7(a) and 7(b) show the Lugol's Iodine (IKI) test on TPS solid medium with growth *Aspergillus* sp. Figure 7(b) is TPS medium with a growth of *Apergillus* sp. Figure (b) is TPS with growth of *Aspergillus sp.* after Lugol's Iodine solution was pour onto it. It clearly shows an appearance of clear zone in the center of the plate indicating amylolytic activity had taken place. Amylolytic activity is an enzymatic splitting of starch into soluble products.

From the results it can be observed that a diameter of 18mm out of 90mm of the petri dish, roughly 20% of the starch, has undergoes amylotic activity during the first week of *Aspergillus sp.* growth. Using TPS solid medium that act as diffential medium that tests the ability of *Aspergillus sp.* to produce exoezymes, α-amylase that hydrolyze starch. Iodine is added to the plate and the Iodine turned purplish in the presence of starch. The clearing around the fungi growth indicates that the organism has hydrolysed starch. Most of the fungi produce enzyme called hydrolases such as α-amylases. Hydrolases catalyse the splitting molecule into smaller molecules in the presence of water (Viens, Lacombe-Harvey, & Brzezinski, 2015). Starch is a polysaccharide made up of α -D-glucose subunits; it consists of two constituents that are amylose, an unbranched glucose polymer and amylopectin, a large branched polymer. The α-D-glucose molecules in both amylose and amylopectin are bonded by 1,4- α –glycosidic linkages. Both amylopectin and amylose are hydrolysed by using α-amylases, to yield monosaccharides (glucose) (Hashim & Sohail).

. Starch is too large to pass through the microorganism cell membrane. Therefore, the fungus must first be split the polysaccharides into smaller fragments or individual glucose molecules according to its metabolic value. The overall reaction is the complete hydrolysis of the polysaccharide to its individual a-glucose subunits. When organisms produce α –amylase that was grown on starch solid medium, they hydrolyze the starch in the solid medium surrounding the growth. Because both the starch and its sugar subunits are soluble in the medium, no differences can be observed; the reagent iodine is used to detect the presence or absence of starch in the vicinity around the bacterial growth. Iodine reacts with starch and produces a dark purple; therefore, any microbial starch hydrolysis will be revealed as a clear zone surrounding the growth.

D. Weight loss of TPS film

The weight is included with the petri dish weight as the weight needs to be kept at a similar condition. The weight of the TPS film is taken nearly every week to determine the weight loss as it indicates the degradation of TPS film by *Aspergillus sp.*Figure 8 is about weight difference. The weight difference for every ten days from the initial day(zeroday) until day 60 is recorded.

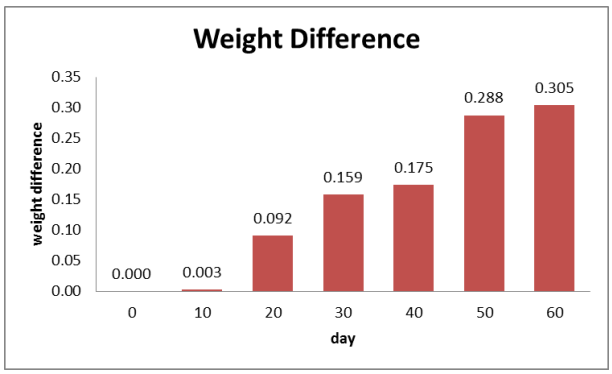

Figure 8: Weight diffence from zero day weight

Figure 9 presents the weight loss of the TPS film, where from the data given the degradation efficiency can be caluclated. From the graph, the initial weight of petri dish including the TPS film is 16.822g and keep on decreasing to 16.818g at day 10, 16.730g at day 20, 16.663 at day 30, 16.647 at day 40 and 16.553g at day 50 and for final observation at day 60 the weight of the petri dish is 16.517g.

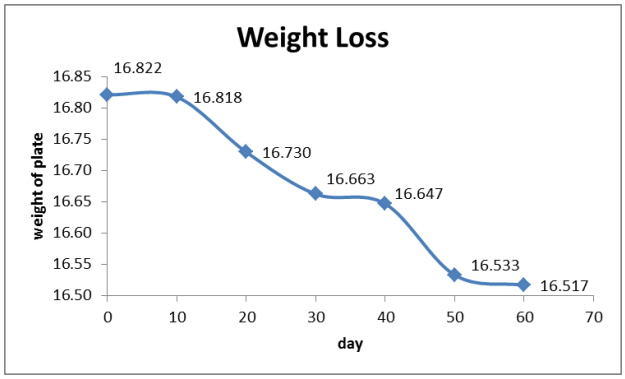

Figure 9: Graph of TPS film weight loss

$$DE\% = \frac{w_0 - w_1}{w_0} \times 100$$

The value of degradation efficiency (DE%) obtained from weight loss method after the initial day(zero day) and final incubation time (60 days) were determined. DE% was calculated using the equation suggested by Housseini et al., 2010 where w_0 and w_1 are the weight loss after initial and final incubation time, respectively.After two months, 1.81% of DE is obtained. Assuming that every month degradation is 0.90% of degradation, 111 months is equivalent to 9.25 years for it to be completely degraded.

During the thermoplastic process, the plasticizer would form a strong interaction between intra- and intermolecular hydrogen bonds with starch for plasticization (Yang, Yu, & Ma, 2006). But as the days goes by the bonds are meant to be broken and undergoes degradation. The degradation mechanisms are held by hydrolysis in a two-step process, depolymerisation and demineralization. Depolymerisation is when the enzyme binds to the polymer substrate and subsequently to demineralization which catalyses a hydrolytic cleavage. Then, it is mineralized it to either one, carbon dioxide and water or carbon dioxide, water and methane under aerobic or anaerobic respectively(Premraj & Doble, 2005).

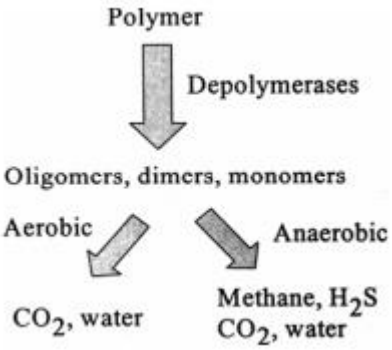

Figure 10: Simple reaction pathway during biodegradation of polymers (Premraj & Doble, 2005)

IV. CONCLUSION

The purpose of the research to identify thermoplastic starch degrading species from organic food waste compost had achieved. Throughout the study, microorganism from the compost was isolated on TPS solid medium to determine its capability to grow on Tacca Leontepooides thermoplastic starch, One species of fungi was selected to continue that study and isolated until it a pure culture was obtained. The morphology and characteristic of the fungal was observed both by naked eyes and under light microscope. From the observation, it was determined that the fungal have similarity with genus *Aspergillus* section Nigri. Several test had done to test the degrading capability of the fungal such as preparing two types of solid medium TPS and TPSAA. Throughout the observation, Aspergillus sp. only grow on TPS while on TPSAA solid medium have no growth even after two months of observation. This is due to low pH in the solid media where *Aspergillus sp.* was vulnerable to the presence of organic acids (acetic acid), which caused cause acidification of the cell.. A clearzone was formed when Lugol's Iodine (IKI) was conducted. It was confirmed that *Aspergillus sp.* was α-amylase producer thus it was able to undergo degradation producing carbon dioxide and water as end products. The color of dark purple appeared when it reacts with starch. A clearing around the *Aspergillus sp.* growth indicates that the organism has hydrolysed starch. The degradation efficiency of TPS film after 2 month is 1.81%. Overall, this research able to determine fungal that able to degrade thermoplastic starch which is *Aspergillus sp*

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