Isolation and identification of *Aspergillus Niger* degrading Thermoplastic Starch from compost soil.

Ain Nadhirah Binti Mohd Fuaad, Suhaila Binti Mohd Sauid

Faculty of Chemical Engineering, Universiti Teknologi Mara

***Abstract*— In this research study, TPS are serves as the bio plastic that need to be degrade by the microbial action. The study was focused on the degradation by Aspergillus sp which are fungi species. The source of the fungi was obtained from the isolation of the fungi sp from the compost soil. Primarily, the microbes were identified and isolated from dilute soil compost. The microbes were streaked and let it grow on the selective media which are PDA agar to identify it as a fungi species. At once, TPS were prepared and let the fungi degrade it. The optimal temperature for the incubation process was 37° C and the duration of the study was two weeks. The identification of fungi sp by morphological characteristics identified that the fungi sp was Aspergillus sp Starch hydrolysis also were done to indicate the presence of the amylase enzyme that were hydrolyze the starch. The presence of amylase will create a clear halos zone surrounding colonies which indicate their ability to digest starch. Next, the biodegradation of TPS film was study based on weight loss of the TPS film. The TPS film showed a decreasing in weight value during the observation. It can be concluded that, *Aspergillus* sp from compost soil are one of the microorganism that are responsible for the thermal plastic degradation.**

***Keywords*— *Tacca leontopetaloides starch, Aspergillus sp,* compost soil, Thermoplastic starch**

1. INTRODUCTION

Since the last few decades, the need of plastics increased drastically since it has applied wide range of application from industrial to daily needs. It has been widely used as packaging app (container and plastic bags), building product (plastic pipe), clothing and medical devices ranging from surgical equipment and blister packs for pills. With more and more plastics being employed in human lives, it will increase the pressure on capacities available for plastic waste disposal. In the long run, plastic is a material that Earth cannot digest since it is made from petroleum. It will be remained for hundreds of years and only will break down into smaller fragments. The degradation of plastic had always been issues since it is related to the environment conditions. The smaller particles of plastic will attract toxic chemical and will be ingested by wildlife on land and ocean which will contaminate the food chain. The growing of environmental pollution caused by synthetic plastic has led to search of alternative material such as biodegradable bioplastic.

Thus, such synthetic polymer will apply renewable sources such as starch, corn and sugar to substitute the polymer so that it can undergo biodegradation process. In addition, the use of bioplastic has attracted the market intention since the cost of the raw material is cheaper, abundant and renewable than raw material for synthetic polymer (Wang, 2017). One of the type of bioplastic is thermoplastic starch film (TPS). TPS itself comprise of starch that are from *Tacca leontopetaloides* which are also known as Polynesian arrowroot starch. It is a species of yam family and geographically distributed from western Africa to Southeast Asia to Northern Australia. *Tacca leontopetaloides* has been reported to be used as stomach ailment and may treat diarrhea, dysentery and internal hemorrhaging in the stomach and colon (Garden, N.T, 2016).

The process of TPS production involved the use of native and slightly modified starch. However, the use of starch itself in bioplastic manufacturing also have its own disadvantages. Starch may cause brittleness to the material if the absence of suitable plasticizer and hydrophilic nature of starch (Nurul Shuhada et.al, 2013). This will affect the mechanical properties of the material since it will deteriorate upon exposure to environmental condition like humidity. To eliminate this drawback, starch itself need to be blended with another synthetic polymer or plasticizer. The most common plasticized is water and it has been used in the

thermal processing of starch based polymer. However, the use of water alone is not preferable because the TPS still will have poor mechanical properties (]Liu,H. et.al 2009). In case, an evaluation towards variety of plasticizer was done and it stated that the plasticizer that are usually being used in plasticization of starch are glycerol, glycol, xylitol, sorbitol, sugars and amide such as urea, formamide and ethylene bis-formamide (Dai, H., et.al, 2009). Plasticizer play a vital role since it could form hydrogen bond with starch which give rise to a strong interaction between intra- and intermolecular hydrogen bond in starch and make starch display the plasticization (Yang et.al, 2006).

Apart from that, the TPS has played a pivotal role especially in reducing the amount of plastic waste because it can be incorporated into soil as organic fertilizer. The process of biodegrading the TPS involving the breaking large molecules into smaller molecules or fragment. The process is responsible by the microorganism and will convert the material into biomass, water and carbon dioxide. The microorganisms that are involved in such process are bacteria, fungi and algae are reported as biological microorganism that degrades plastic naturally. (Rutkowska M,et.al,2002)*Pseudomonas,Mycobacterium,Corynebacterium,Aeromonas,Rhodococcus, Bacillus* (Koutny M, et.al,2009). *Cladosporium, Aspergillus, Penicillinum* and *Fusarium* are the example of the microorganism that are responsible in biodegradation.

This study involved the isolation and identification of fungi that are responsible for the degradation of thermal plastic starch from compost soil, Fungi will secrete amylase enzyme to hydrolyze the starch. The secretion of amylase enzyme can be tested through starch hydrolysis test. The biodegradation of TPS study can be demonstrate by analyze the weight loss of the TPS film. In this study, the weight loss was study by growing the fungi on the TPS film and study the weight loss after two weeks of incubation.

2. MATERIAL AND METHODOLOGY

2.1 Preparation of TPS solid media and Acetic Acid TPS solid media.

Tacca leontopetaloides starch, glycerol and 5% acetic acid was suspended into 1 liter distilled water by following the composition in Table 1. It was heated and stirred until it became homogenized. Then, the molten TPS mixture was autoclaved and left to cool until approximately 80° C. After that, the solid media was poured onto petri dishes and stored inside chiller.

For TPS with glycerol, *Tacca leontopetaloides* starch, and glycerol was suspended into 1 liter distilled water by following the composition in Table 1. It was heated and stirred until it became homogenized. The next processes were same as the preparation of TPS with acetic acid solid media which the molten TPS mixture was autoclaved and left it cool. The solid media then was poured onto petri dish and stored it inside the chiller.

Table 1: The composition of TPS agar

Agar Coded	**Distilled water (%) from 1 L**	**Glycerol (%) from 1 L**	**5% Acetic Acid (%) from 1 L**	***Tacca leomtepeloides (%) from 100 grams***
TPS/GLY	90	10	-	20
TPS/ACE	80	10	10	20

2.2 Isolation of the fungi

1 gram of soil was taken at landfill area Seremban, Negeri Sembilan was transferred into 10 ml of distilled water and mixed well. The mixture undergoing serial dilution until $1/10000^{th}$ dilution. The last dilution was streaked on TPS solid media and incubated for 24 hours. Any microorganisms that grown on the TPS solid media were then transferred to PDA agar for isolation and identification.

2.3 Observation of Apergillus sp

During preparation of smear, a drop of sterilize water was added on the slide. A small sample of single colony was inoculated from the plate and stirred it gently with water on the slide. Next, let the smear dry by passing through the flame of a Bunsen burner two to three times with the smear side up. Then the smear was observed under microscope.

2.4 Starch Hydrolysis Test

An inoculum from a pure culture was spread on a sterile plate of TPS solid media. The inoculated plate is incubated at 35-37 C for 24 hours. Lugol iodine was then added to flood the growth. After that, clear halos surrounding the colonies was observed to indicate the presence of alpha amylase enzyme.

2.5 Growth of *Aspergillus sp* on TPS starch film

Table 2: Composition of TPS film

Agar Coded	Distilled water (ml)	Glycerol (ml)	5% Acetic Acid from (ml)	*Tacca leomtepeloides* (grams)
TPS/GLY	80	10	5	20

Starch was boiled with water, glycerol and acetic acid and vigorously mechanically stirred at 150° C until the suspension become transparent. Then, the suspension was cooled down and a starch-plasticizer paste was obtained. The paste was poured into a thin layer container and dried in the oven to a constant weight at 50° C for 24 hours. A thin layer thermoplastic starch (TPS) with 2 mm thickness formed was cut into small size (2cm x 2cm) for further testing. The composition of formulated TPS is shown in Table 2.

Next, 10 ml of distilled water was flooded onto the PDA agar that has 24 hour *Apergillus sp* growth on to collect the spore. Then. 1 ml of spore suspension and flood onto 2cm x 2cm TPS film. Then, the film was incubated for and the growth of the *Aspergillus sp* at 37 °C.

3. RESULTS AND DISCUSSION

3.1 ISOLATION OF FUNGI

After the fungi was isolated on the PDA, the fungi *sp* then were growth on TPS with glycerol and TPS with acetic acid solid media. Within 3 days, the *Aspergillus sp* was growth on the TPS agar. However, there were no growth on the TPS acetic acid agar as shown as figure 2 below. Fungi strain showed the highest growth at temperature between 20 to 37 °C and pH level between 4 to 6.5 are optimal. The incubation temperature for the experiment was 37°C. Therefore, it was a preferable temperature for the growth of Aspergillus sp. According to Eric Camp from the website, the pH for 5% acetic acid is 2.4 (Camp, 2017). Hence, the condition of the agar is too acidic for the growth of the fungi sp. The source of culture probably dead since the condition for growth was not preferable.

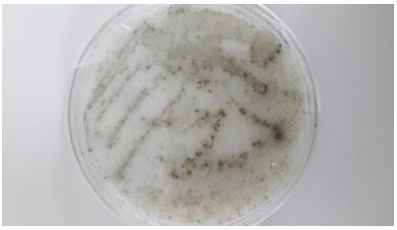


Figure 1 The growth of fungi strain on TPS with glycerol solid media.

Figure 2 Absence growth of fungi strain on TPS with acetic acid solid media.

3.2 OBSERVATION OF FUNGI SP

Under the light microscope, the fungi sp was viewed as the figure 3. The microorganism was confirmed as *Aspergillus sp* by using morphological characteristics. Microscopic morphology of *Aspergillus niger* showing

large, globose, dark brown conidial heads, which become radiate, tending to split into several loose columns with age (K Diba,et.al, 2007).

Conidiophores are smooth-walled, hyaline or turning dark towards the vesicle. Conidial heads are biseriate with the phialides born on brown, often septate metulae. Conidia are globose to subglobose, dark brown to black and rough-walled (K Diba et.al, 2007). The morphological characteristic stated were same with the figure 4. From figure 4, there are conidial heads in brown colour and the conidia are globose to subglobose shape and have a rough walled.

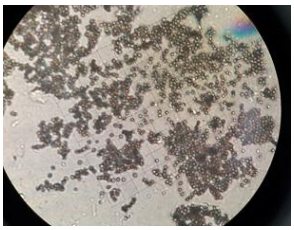


Figure 3 Aspergillus niger under 40X magnification

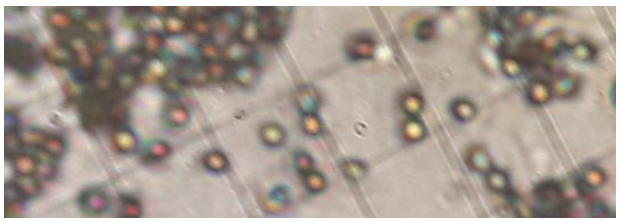


Figure 4 Zoom Image of the Aspergillus niger under 40 X magnification

3.3 STARCH HYDROLYSIS TEST

The purpose starch hydrolysis test is to see if the microbe can use *starch*, a complex carbohydrate made from glucose, as a source of carbon and energy for growth. Use of starch is accomplished by an enzyme called alpha-amylase. After inoculation and overnight incubation, iodine reagent is added to detect the presence of starch such in figure 5. Iodine reagent complexes with starch to form a blue-black color in the culture medium. From figure 5, there are presence of clear halos surrounding colonies after 24 hours incubation. This indicate their ability to digest the starch in the TPS solid media due to the presence of alpha-amylase. α-amylases (endo-1,4-α-D-glucan glucohydrolase) are extracellular enzymes that randomly cleave the 1,4-α-D-glucosidic linkage between adjacent glucose units inside the linear amylose chain (A. Pandey,et.al, 2005)

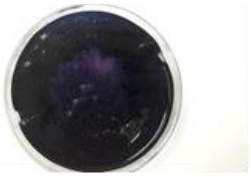
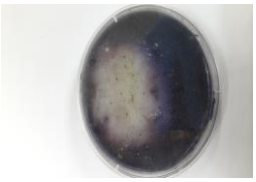
After Flooding	After 24 hours
	

Figure 5 Starch Hydrolysis Test on Aspergillus niger

3.4 GROWTH ON *ASPERGILLUS NIGER* ON TPS STARCH FILM

. The initial weight of sterile TPS film are 0.8812g and the colour of the TPS film was milky white and opaque. At day 1, the fungi were spread on the 2cm x 2cm TPS film at let it growth. During day 2, there were some changes detected on the TPS starch film. There were growth of *Aspergillus niger* on the films. There was spore's formation on the surface on the TPS film. The color of the TPS film was change from milky white to yellowish. Figure 6 showed the physical condition of TPS film after 16 days of incubation. It can be seen opaque surface changes to transparent. The color of the TPS film become dark yellowish. Apart from that, the weight of the TPS film was decreased to 0.6289 g. The weight loss indicated that the TPS film has been biodegrade by *Aspergillus niger*.

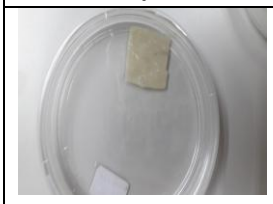
Day 1	Day 2
	

Figure 5 Aspergillus growth on TPS starch film

Figure 6 The 16th days of incubation

4. CONCLUSION

The experiment considered successful because overall objectives for the research project were all achieved. Fungi that was isolated from the compost soil was identified as *Aspergillus niger* because of the similarity with the morphological characteristic. Apart from that, the *Aspergillus niger* secreted amylase since there were presence of clear halos zone on the TPS solid media. It can be concluded that, *Aspergillus niger* was confirmed as one of the responsible microorganism that degrade the TPS film.

ACKNOWLEDGMENT

I would like to take this opportunity to express my profound gratitude and deep regard to my supervisor Madam Suhaila Binti Mohd Sauid for her exemplary guidance, valuable feedback and constant encouragement throughout the duration of the project. Her valuable suggestions were of immense help throughout my project work. Her perceptive criticism kept me working to make this project in a much better way. Working under her was an extremely knowledgeable experience for me.

I thanked to Encik Irwan Bin Zainuddin as the Assistant Engineer for Industrial Biotechnology Laboratory, University Technology Mara for the equipment guidance and support. Lastly, I would also like to give my sincere gratitude to my friends Nabila Huda and Arifah Rashidi who were also my team mate in which without them, the research would be incomplete.

REFERENCES

[1] Ying Zheng, Ernest K. Yanful & Amarjeet S. Bassi, "Critical review in Biotechnology," *A review of plastic waste biodegradation,* pp. 243-250, 2005.

[2] Rutkowska M, Heimowska A, Krasowska K, Janik H, "Biodegradability of Polyethylene Starch Blends in Sea," *Pol J Environ Stud,* pp. 267-274, 2002.

[3] Koutny M, Amato P., Muchova M, Ruzicka J,, " Soil bacterial strains ableto grow," *Int Biodeterior Biodegrad,* pp. : 354-357, 2009.

[4] Katarzyna Leja, Grażyna Lewandowicz, "Polymer Biodegradation and Biodegradable Polymer," *Polish J. of Environ. Stud,* pp. 255-266, 2010.

[5] Sanchez J, Tsuchii A, Tokiwa Y, " Degradation of polycaprolactone at 50°C by a thermotolarant Aspergillus sp.," *Biotechnol Lett ,* p. 849–853., 2000.

[6] Ezgi Bezirhan Arikan and Havva Duygu Ozsoy, "Investigation of Bioplastics," *Journal of Civil Engineering and Architecture,* pp. 188-192, 2015.

[7] Garden, N.T, "NTGB," 13 November 2016. [Online]. Available: http://www.ntbg.org/plants/plant_details.php?plantid=10965.

[8]]Liu,H., Xiea,F., Yua, L., Chena, L., and Lia, L, " Thermal processing of starch-based polymers," *Progress in Polymer Science,* p. 1348, 2009.

[9] Dai, H., Chang, P.R., Geng, F., Yu, J., and Ma, X, "Preparation and Properties of Thermoplastic Starch/Montmorillonite," *Additive Journal Polymer Environment,* pp. 225-232, 2009.

[10] Yang, J.H., Yu, J.G. and Ma, X.F., "A Novel Plasticizer for the Preparation of Thermoplastic Starch," *Chinese Chemical Letter,* pp. 133-136, 2006.

[11] E. Camp, "Weak Acid - Tirtation of Acetic Acid," 1 June 2017. [Online]. Available: ttps://depts.washington.edu/chem/facilserv/lecturedemo/pHofAceticAcid-UWDept.ofChemistry.html.

[12] K Diba, Kordbacheh P, Mirhendi SH, S Rezaie, M Mahmoudi, "Identification of aspergillus species," *Pakistan Journal of Medical Science,* pp. 867-872, 2007.

[13] H. Anto, U.B. Trivedi, K.C. Patel, "Glucoamylase production by solid-state fermentation using rice flake manufacturing waste products as substrate," *Bioresource Technology,* pp. 1161-1166, 2006.

[14] A. Pandey, C. Webb, C.R. Soccol, C. Larroche, "Enzyme Technology," *AsiaTech Publisher,* p. 197, 2005.

[15] Nurul Shuhada Mohd Makhtar, Miradatul Najwa Muhd Rodhi, Mohibah Musa, and Ku Halim Ku Hamid, "Thermal Behavior of Tacca leontopetaloides Starch-Based Biopolymer," *International Journal of Polymer Science,* pp. 1-7, 2013.

[16] H. C. a. L. Wang, Technologies for Biochemical Conversion of Biomass, London: Academic Press, 2017, pp. 1405-1408.