

## Colonization of Plastic by *Xylaria* sp\*.

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Studies were conducted to show physical evidences of colonization of polyethylene plastic strips by *Xylaria* sp. using scanning electron microscopy. Optimum environmental conditions for growth of the fungus such as temperature and hydrogen ion concentration were determined by growing the organism on different mineral media with different substrates like tannic acid, gallic acid, filter paper and cellulose as carbon sources. The fungus grew best at 25°C and at pH 5. When grown at these optimum conditions, growth was vigorous on mineral medium with 0.5% glucose and plastic strips as co-carbon source. The fungus produced mucilaginous sheath that facilitated adhesion of mycelial growth on the surface and edges of the plastic strips. The strips became embedded in the mycelial mat after 50 days of incubation. Scanning electron microscopy showed visible damages of the surface structure of the plastic strips. There were tearing and striations caused by active burrowing of *Xylaria* hyphae on the polyethylene material.

**Key words:** plastic materials, enzymes, optimum conditions, scanning electron microscopy, carbon source

Plastic bags and wrappers have been a major component of our contemporary way of living. These plastics are made of high molecular weight, semi crystalline polymer prepared from ethylene. The ethylene monomer is prepared in large quantities by the cracking of natural gas and light petroleum (Knapczyk & Simon 1992). The wide use of plastics is attributed to their durability, chemical resistance and stability. However, these very qualities are also the factors that present a problem in their ultimate disposal (Cain 1992). Plastics have very slow phase of degradation, making it a growing source of public concern in recent years. In the Philippines, plastic bags and wrappers end up in canals and waterways that clog the drainage. As such, they cause major floods during the rainy season.

There are several disposal methods for plastics. Among these methods are incineration, recycling and photo degradation. Some systems employ auto oxidant

additives to form peroxides that initiate the breakdown of the polymer chain making it susceptible to microbial attack (Albertson & Rantky 1976). Studies that involve the use of biological factors in the degradation of these synthetic materials have been conducted. The ultimate aim is to reduce the plastics to environmentally innocuous materials. One of these processes is biodegradation accomplished through the action of microorganisms that break down the polymer molecules themselves. The microorganisms could attack susceptible filler material present in the polymer such as starch (Knapczyk & Simon 1992). However the most important process causing bio deterioration of plastics is assimilatory process in which constituents of the plastic are used as sources of carbon by microorganisms (Egginis et al. 1971).

Numerous microorganisms are reported as active decomposers of plant substrates such as cellulose and lignin. This ability of microorganisms to decompose natural polymers makes them important agents of decomposition of synthetic bio polymers such as plastics. For the past decade, several species of fungi such as *Chaetomium globosum*, *Trichoderma* sp.,

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*Penicillium funiculosum* and *Aspergillus niger* are reported to have plastic biodegradative ability. Cuevas & Manaligod (1997) reported a fungal isolate consisting only of sterile melanin pigmented mycelia growing on sando plastic bag buried in forest soil and litter in the lowland secondary forest of Mt. Makiling, Laguna. The fungal isolate was able to grow in mineral medium with 0.5 percent glucose and plastic strips as sources of carbon. In this medium the plastic strips decreased in weight indicative that the fungus was able to use carbon from plastic. The fungus is therefore a potential decay agent of polyethylene plastics. Initially reported as ascomycete sterile dark mycelia (ASDM), further cultural studies of the isolate showed that it belongs to genus *Xylaria* under Order Xylariales, Class Ascomycete (unpublished data).

The polymer degrading ability of such microorganisms is attributed to the enzyme systems they possess. Thus, setting the right conditions for enzyme induction would lead to a high decay ability of the organisms. These present studies were conducted to determine the optimum physical conditions that favor cellulose and lignin decomposition that will also favor plastic degrading ability of the fungal isolate. An attempt was also made to determine the mode of attack of the isolate in the degradation process of polyethylene plastic sheets in vitro using scanning electron microscopy.

## Materials and Methods

### Media used

The isolated mycelium was cultured in potato dextrose agar (PDA) slants with 100 ppm streptomycin solution to prevent bacterial contamination. The culture was preserved by incorporating sterile mineral oil into the slants and kept in a glass cabinet in an air-conditioned room. It was deposited to the culture collection of decomposer fungi of Dr. Virginia C. Cuevas. Whenever the isolate is needed, a PDA subculture is made from the preserved mineral oil culture.

To test the ability to degrade cellulose, two types of media were used: filter paper mineral medium (filter paper as a source of native cellulose) and carboxy methyl cellulose medium (carboxymethyl cellulose, CMC, as a source of partially degraded cellulose). Filter paper mineral medium consisted of the following: 5.0 g ammonium tartrate, 1.0 g Difco malt, 0.5 g  $MgSO_4 \cdot 7H_2O$ , 0.01 g  $CaCl_2 \cdot 2H_2O$ , 0.1 g NaCl, 0.01 g  $FeCl_3$ , 0.02% Tween 80, 5.0 ml 1% Thiamine and Whatman filter paper #1 in 1000 ml distilled water. 1ml of trace element solution with the following composition was added to the mineral medium - 5 g/L  $FeSO_4$ , 1.6g/L

$MnSO_4 \cdot 7H_2O$ , 1.4 g/L  $ZnSO_4 \cdot 7H_2O$ , 2.0g/L  $CaCl_2$ , 1000 ml distilled water. Carboxy methyl cellulose medium (Leisola and Kauppinen 1978) consisted of the following: 0.2 %  $KH_2PO_4$ , 0.03%  $MgSO_4 \cdot 7H_2O$ , 0.14%  $(NH_4)_3HPO_4$ , 0.03%  $CaCl_2 \cdot 2H_2O$ , 0.07% Carboxy methyl cellulose, 0.02% Tween 80, 1000 ml distilled  $H_2O$ .

Tannic and gallic acid media were used to assay potential lignin degradation through production of phenolic oxidases (Davidson 1938). The two media were prepared in this manner. 20 g agar and 15 g Difco malt were dissolved in 850 ml distilled water and sterilized. 50 g tannic acid were dissolved in 150 ml sterile distilled water. This solution mixed with the 850 ml sterile agar solution when the agar has sufficiently cooled gave a 5% tannic acid solution. (Heating tannic and gallic acid solutions with agar causes hydrolysis of agar, therefore the acid solutions and the agar should not be autoclaved together). The resulting medium was milky white. Gallic acid medium was prepared in the same manner. 5 percent gallic acid was used. However gallic acid did not change the color of the malt agar.

### Optimization of physical conditions for degradation of bio polymers

#### 1. Temperature

To determine the optimum growth soil temperatures in the forest where the organism was isolated were taken daily for one week at dawn and noon. The mean temperature readings were taken as the temperature regimes where the organism was cultured.

##### a. Growth on filter paper and carboxy methyl cellulose

In the filter paper test, two triplicates (Sets A & B) of petri dishes containing circular shaped Whatman # 01 filter paper fitted to the bottom plate, with 15 ml / petri dish mineral medium, were prepared. Each plate was inoculated with a loopful of the mycelium of *Xylaria* sp. at the center of the filter paper. After inoculation set A plates were incubated for seven days at 25°C and set B at 37°C. The diameter of the mycelial growth was measured after 7 days incubation.

In the carboxy methyl cellulose (CMC) test, triplicate of flasks containing 50 ml of cellulose medium was inoculated with the organism. CMC induces the production of cellulose particularly endo-1,4  $\beta$ -glucanase (C<sub>1</sub>). The flasks were then incubated for seven days at 25°C and another set at 37°C. After incubation each mycelial biomass produced was removed from the culture, oven dried and weighed.

##### b. Tannic acid and gallic acid utilization

Each tannic and gallic acid plate was inoculated

at the center with a loopful of *Xylaria* sp. mycelia. The plates were incubated at 25°C and at 37°C for seven days. Three replicate plates were done for each treatment. The reaction of *Xylaria* sp. to gallic acid and tannic acid media were recorded using (+) and (-) sign to indicate the extent of the browning of the culture media with respect to their control set-ups.

## 2. pH

The same sets of media used for determining optimum temperature were prepared to determine most preferred pH in triplicates. All plates and flasks were incubated for seven days at temperature where growth was based on the previous results on temperature tests. The same methods used for measurement of growth in temperature tests were followed.

## 3. Growth of *Xylaria* sp. in the presence of polyethylene plastic strips

After the results of the studies on temperature and pH were obtained, the test on the ability of the organism to grow on medium with plastic strips as co-source of carbon was done. The temperature and pH where the organism grew best was used in the test.

Two sets of flasks containing 50 ml mineral medium supplemented with 0.5 percent glucose were prepared, each set having five replicates. pH was adjusted to five. Ten pieces of polyethylene plastic strips cut into 1 x 1 cm were added to the first set and were then inoculated with a loopful of *Xylaria* sp mycelium. The second set without polyethylene plastic strips were used as control. All the sets were then incubated for fifty days at 25°C in an air-conditioned room, which varies from 25°C during daytime to 28°C during nighttime.

At the end of the incubation period, plastic strips were removed from the culture medium and prepared for scanning electron microscopy. This was done to study the extent of colonization and possible degradation of the plastic by the organism.

# Results and Discussion

## Temperature Optimization

The organism was grown at temperatures 25°C and 37°C. These temperatures were the means obtained from repeated measurements of soil temperatures. The ability of the fungus to degrade cellulose and lignin was tested at these two temperatures.

Table 1 presents the results of the tests on growth at different carbon sources at two incubation temperatures. No growth was observed in the filter paper at 37°C, while the fungus grew normally at 25°C in the same medium. There was no significant difference in mean biomass production in CMC at 25°C and at 37°C. These results imply that the degradation of native cellulose, in this case filter paper, was greatly hampered by temperatures close to 37°C. In contrast the fungus can still utilize partially degraded cellulose at this temperature.

The growth of the test organism on gallic acid and tannic acid at 25°C was much higher than at 37°C. Utilization of the acids was indicated by the deep browning of the surrounding medium extending from center of the colony to the margin of the fungal mat. Darkening of the medium was very intense at 25°C. The plates incubated at 37°C using tannic acid in the medium showed no reaction, while those plates containing gallic acid showed a mild reaction. A slight browning was formed just beneath the point of inoculation and was only visible at the underside of the dish.

It has been proposed that the sequence of lignin degradation begins with demethylation to yield diphenolic acids that has two adjacent hydroxyl groups. The rings are then opened up by deoxygenase enzymes to expose the aliphatic chain attached to the lignin. The aliphatic chains are then split off from the polymer by an oxygenase enzyme such as laccase. The aliphatic side-chains are similarly degraded by oxidases. However, the tests for gallic acid and tannic

Table 1. Growth\* *Xylaria* sp. in filter paper, carboxy methyl cellulose (CMC), gallic acid and tannic acid at two incubation temperatures.

Carbon source	Parameters measured	Incubation Temperatures	
		25°C	37°C
filter paper	colony diameter (mm) *± SD	8.67 ± 1.53	0
CMC	biomass (mg) *± SD	89.7 ± 17.42	59.9 ± 25.88
gallic acid	browning of medium**	++	+
tannic acid	browning of medium**	+++	no change

\*Mean of 3 replicates

\*\*Scoring system used to evaluate reaction to tannic acid and gallic acid media

+ Diffusion zone light to dark brown, formed at the point of inoculation and visible only at the underside

++ Diffusion zone light dark brown formed under most of the mat but not extending to margin of mat; visible from under side only

+++ Diffusion zone light dark brown, extending a short distance beyond the margin of the fungal mat; also visible from the upper side

Table 2. Growth\* (mm) of *Xylaria* sp. in various carbon sources at varying pH of growth medium.

pH of media	Carbon Source			
	Filter paper*	CMC**	Tannic acid***	Gallic acid***
3.0	0.00 <sup>a</sup>	100.47 <sup>b</sup> ± 11.21**	--	--
5.0	33.33 <sup>a</sup> ± 5.03	103.33 <sup>b</sup> ± 23.49	+++	+++
7.0	13.00 <sup>b</sup> ± 1.15	106.93 <sup>b</sup> ± 8.70	+++	++

\*Mean growth diameter (mm) ± SD with three replicates.

\*\*Mean biomass production (mg) ± SD with three replicates.

[Means in a column with the same superscript are not significantly different at 5% level by DMRT]

\*\*\*Scoring system used to evaluate reaction to tannic acid and gallic acid media.

-- no growth

++ Diffusion zone light to dark brown formed under most of the mat but not extending to margin of mat; visible from under side only

+++ Diffusion zone light to dark brown, extending a short distance beyond the margin of the fungal mat; visible from upper side

++++ Diffusion zone light to dark brown, opaque, extending considerable beyond the margin of the fungal mat.

acid utilization do not specify which enzyme/s is/are actively produced by the test organism, it is just indicative of potential production of phenol oxidizing enzymes (Davidson 1988).

These results suggest that this organism may be a cellulose and lignin decomposer. It can grow in both native and partially degraded cellulose as shown by its activities on filter paper and CMC. Likewise, the organism has the potential to produce phenol oxidases, the initial enzymes needed for the degradation of lignin. The organism preferred 25°C - 28°C temperature for its metabolic activity.

#### 2. pH Optimization

Optimization of H<sup>+</sup> concentration was done to determine the optimum pH for growth and production of cellulases and phenol oxidases. Enzyme production was measured indirectly through the measurement of the diameter of growth and mycelial biomass production. Measurement of these two parameters was indicative of the ability of the fungus to utilize substrates and convert them metabolically to microbial tissues through the use of their enzyme systems. Results on the tests on growth of *Xylaria* sp. on different media adjusted to various pH are presented in Table 2. Growth in filter paper was best at pH 5. Analysis of variance test (ANOVA) showed that mean growth on filter paper at pH 5 and pH 7 were significantly different.

Mycelial biomass production in CMC was not significantly different at pH 3, 5 and 7, although the lowest mycelial biomass was produced at pH 3 and highest at pH 5.

Growth was consistently inhibited at pH 3 with tannic and gallic acid as carbon sources. The results seemed to indicate that the enzymes produced for the degradation of cellulose and lignin-like substrates are optimally active at pH 5. This could be reflective of environmental condition of their habitat since the organism was isolated from the forest floor which has a slightly acidic condition.

Plates containing mineral medium with filter paper adjusted to pH 5 caused a change of color of filter

paper from white to pink noted on the 6th day of incubation. This could be indicative that production of enzymes used to degrade the filter paper was high at pH 5 thus yielding a much thicker growth of the mycelia. No such change in color of filter paper was noticed in other cultures.

Figure 1 shows the growth of *Xylaria* sp. in various carbon sources - filter paper, tannic acid, gallic acid and carboxy methyl cellulose, at pH 5 and incubated at 25°C - 28°C for 7 days.

#### Growth of *Xylaria* sp. on mineral medium with polyethylene plastic strips and 0.5% glucose

Figures 2a and b show the contrast in the growth of *Xylaria* sp. in optimal and non-optimal temperature and pH conditions. The fungus in Fig. 2a was grown at 29°C - 31°C and at pH 6.6. Under these conditions of Fig. 2a, the fungus caused 5 mg decrease in dry weight of the plastic strips (Cuevas & Manaligod 1997). In Fig. 2b, the organism was grown at 25°C - 28°C and at pH 5. It was observed that the organism grew much more profusely compared to growing in non-optimal conditions.

No attempt was made to measure the change in weight of the plastic strips when the fungus was grown in optimal conditions. It was very difficult to remove the mycelia since the fungus closely adhered to the plastic strips and grew into it. The initial growth of the fungus in the flasks containing mineral medium with 0.5 percent glucose and plastic growth may be attributed to the glucose present in the medium (Cuevas & Manaligod 1997). Thus when the glucose was depleted, the organism had to utilize the plastic strips as source of energy. The good growth of the organism in this medium in Fig. 2b can only be attributed to its capability of the organism. According to Johnson et al. 1993, organisms growing on plastic materials may either utilize the plasticizer molecule (i. e. starch, cellulose) or the polymer molecule. In this study it can not be determined which components of the plastic material was used by the fungus for growth.

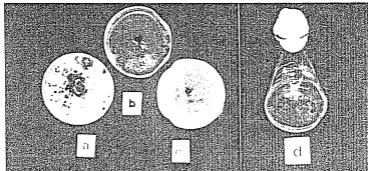


Figure 1. Growth of *Xylaria* sp. in: (a) filter paper, (b) tannic acid, (c) gallic acid and (d) carboxy methyl cellulose at pH 5 and incubated at 25 °C – 28 °C for 7 days.



Figure 2a. Growth of *Xylaria* sp. in mineral medium with 0.5% glucose and plastic strips; pH of medium = 6.6 and incubation temperature at 29 °C – 31 °C for 50 days

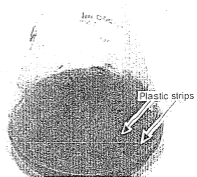


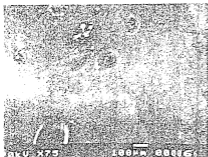
Figure 2b. Growth of *Xylaria* sp. in mineral medium with 0.5% glucose and plastic strips; pH of medium = 5.0 and incubation temperature at 25 °C – 28 °C for 50 days.

More sophisticated method should be done to ascertain this such as comparative chemical analyses of colonized materials and the original uncolonized plastic strips.

On the third day of incubation, a mucilaginous sheath was observed which started at the edges of the plastic strips extending over its surface. At the end of the 50-day incubation period, mycelial growth of the organism was observed enveloping the plastic strips. Other researchers like Sietsma et al. 1981, Whitekettle 1991 and Milstein et al. 1992 mentioned that the active colonizers of polymer are able to adhere to their substrates because of their ability to produce exocellular polymers composed primarily of nonionic and anionic polysaccharides. Such adhesion to

surfaces of substrates is a decisive step in microbially induced corrosion. Kaeppli & Fiechter (1976) observed that hydrocarbon utilization by microbial cells first involves a passive adsorption to lipophilic lipopolysaccharide of the cell surface to the alkane group of the polymer. It was hypothesized that after adhesion, solubilizing agents are produced and secreted by many microorganisms capable of utilizing water-immiscible compounds (Reddy et al. 1982, Gutnick & Minas 1987).

As *Xylaria* sp. grew on mineral medium, polyethylene strips was observed to be deeply embedded on the mucilaginous sheath formed by the organism. Favorable environmental conditions such as optimum incubation temperature and right pH must have



**Figure 3a.** Scanning electron microscope image of the surface of the plastic strip in the mineral culture medium without the fungus (control).



**Figure 3b.** Scanning electron microscope image of the surface of the plastic strip incubated in the mineral culture medium with profuse growth of *Xylaria* sp.



**Figure 4a.** Tearing of plastic surface as shown in SEM. Arrow A shows a hypha in actual process of burrowing. Arrow B shows a hypha rising from a torn surface.



**Figure 4b.** Evidence of degradation revealed by SEM. Striations (pointed by arrows) on the component of plastic surrounding the hypha.



**Figure 4c.** Undersurface of the plastic strip as seen in SEM. Arrow points an actively burrowing hypha on the edge of the material.



**Figure 4d.** Formation of capsular material outside the hypha.

facilitated the production of extra cellular mucilage by the test organism. Note that in Fig. 2a the plastic strips were clearly identifiable since little mucilaginous sheath was produced. In Fig. 2b, the plastic strips were barely seen since the strips were completely enveloped by the dark mycelia of *Xylaria* sp.

#### Verification of Colonization of the Plastic Strips by Scanning Electron Microscopy

Mycelia of the fungus on polyethylene after sufficient adaptation to the conditions in the set-up penetrated into the plastic material. Some chemical reactions might have taken place since evidences of bio-corrosion by the mycelia observed through scanning electron microscope were tearing, pitting and striating of the plastic materials. Figures 3a and b show the scanning electron microscope (SEM) image of the surface of the plastic strips incubated with and without the fungus. Other SEM data for the colonized polyethylene plastics are shown in Figures 4a - d.

#### Summary and Conclusion

Prolonged growth of mycelia of *Xylaria* sp. was observed in mineral medium at pH 5.0 with 0.5 percent glucose and plastic strips as sources of carbon and incubation temperature of 25°C - 28°C. The plastic strips were completely enveloped by the mycelia of the fungus after seven weeks incubation. Scanning electron microscopy of the plastic strips showed that fungal mycelia colonized the strips. The hyphae of the fungus were observed burrowing on the edges of the plastic strips. Tearing and shearing of the surface of the plastic strips were also seen. However this study only presented evidences that the fungus is capable of colonizing plastic strips but did not identify whether the polymer components or the plasticizer molecules of the plastic materials were utilized by the fungus for their growth.

#### References

Albertson AC & Ranby B. 1976. Proceedings of the 3rd International Biodegradation Symposium, Rhode Island p. 743. Applied Science Publishers, London.

Cain RB. 1992. Microbial Degradation of Synthetic Polymers, pages. 293-339. In: Fry, J.C., Gadd, G.M., Herbert, R.A., Jones, C.W. and Watson-Craik, I.A. (Editors). Microbial Control of Pollution. Society for General Microbiology Symposium 48. University of Cardiff, Cambridge.

Cuevas VC & Manaligod RL. 1997. Isolation of decomposer fungi with plastic degrading ability. Philippine Journal of Science. 126:117-130.

Davidson RW. 1938. Differentiation of wood decaying fungi by their reactions on gallic and tannic acid medium. Journal of Agricultural Research. 57(9):683-695.

Eggs H OW, Mills J, Holt A & Scott G. 1971. Biodegradation and biodegradation of synthetic polymers, pages 267-279. In: Sykes, G. & Skinner, F.A. (Editors). Microbial Aspects of Pollution. The Society for Applied Bacteriology Symposium. Series no.1. Academic Press, London-New York.

Gutnick DL & Minas W. 1987. Perspective on Microbial Surfactants, Biochemical Society Transactions, 15 (Suppl.), 22S-35S.

Johnson KE, Pometo III AL & Nikolov ZL. 1993. Degradation of Degradable Starch-Polyethylene Plastic in Compost Environment, Applied and Environmental Microbiology, 59:1155-1161.

Leisola M & Kauppinen V. 1978. Automatic assay of cellulase activity during fermentation. Biotechnology and Bioengineering. 20:837-846.

Kaeppel O & Feichter A. 1976. The mode of interaction between the substrate and cell surface of the hydrocarbon-utilizing yeast *Candida tropicalis*. Biotechnology and Bioengineering. 18, 967-74.

Knapczyk JK & Simon RHM. 1992. Synthetic Resins and Plastics, pages 623-704. In: Kent, J. A. (Editor). Riegel's Handbook of Industrial Chemistry. 9th ed., Van Nostrand Reinhold, New York.

Milstein O, Gersonde R, Huttermann A, Chen MH & Meister JJ. 1992. Fungal Biodegradation of Lignopolystyrene Graft Copolymers. Applied and Environmental Microbiology. 58:3225-3232.

Reddy G, Singh HD, Roy PK & Baruah JN. 1982. Predominant role of hydrocarbon solubilization in the microbial uptake of hydrocarbons. Biotechnology and Bioengineering. 24:1241-69.

Sietsma JK & Wessels JGH. 1981. Solubility of (13)-b/ (16)-b- glucan in fungal walls importance of presumed linkages between glucan and chitin. Journal of General Microbiology. 125:209-212.

Whitekettle WK. 1991. Effect of surface-active chemicals on microbial adhesion. Journal of Indian Microbiology. 7:105-116.