# Scanning electron microscopy (SEM) investigation of polystyrene damage due to colonization by locally isolated *Xylaria sp.*

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RESEARCH ARTICLE

**Abstract**

**Background and Objectives**: Colonization of microorganisms on pollutants is the first indication of the potential ability of microbes to utilize plastic pollutants as a carbon source by sequential biodegradation into usable form for sustenance. The Philippines is considered as the third highest country contributing to global mismanaged plastic waste. To locally manage and find a natural and innovative solution to this worldwide concern, this study aims to evaluate the capacity of Xylaria sp. SDM (sterile dark mycelia) wild type, which was previously reported to colonize polyethylene plastic, and mutant strains to colonize polystyrene, which is among the widely produced plastic pollutants in the world. Assessment of the ability of local Xylaria sp. strains to grow, penetrate, and damage the surface and inner structures of polystyrene was investigated using scanning electron microscopy (SEM).

**Methods:** Xylaria sp. strains were cultured in a pH 5.0 mineral medium with 0.5% glucose as carbon source and polystyrene as a co-carbon source, and stored at 250C for 50 days. At the end of the incubation period, due to irremovable fungal strains on the surface of the polystyrene strips, samples of polystyrene from each strain were subjected to SEM.

**Results:** On the 20th day of incubation, the presence of mucilaginous sheaths and fungal growth were observed on the surface of treated polystyrene strips. At the end of 50-day incubation period, scanning electron microscopy (SEM) confirmed fungal growth and colonization, through the presence of mycelial mats and hyphae, of the wild type and mutant strains on the surface and subsurface structure of polystyrene except the control. Moreover, physical surface damage in the form of holes, cracks, and crevices on polystyrene demonstrated the active burrowing action of Xylaria sp. strains further supporting the potential of this fungus to damage polystyrene plastic.

**Conclusion:** Whereas fungal growth on a polymer surface is necessary but not sufficient to conclude the process of carbon assimilation as the final biodegradation step, the initial colonization of Xylaria sp. strains on polystyrene supports its ability to establish itself and physically damage the pollutant. Hence, this study extends the existing knowledge on the colonizing ability of Xylaria sp. on plastic making it a potential candidate organism to biodegrade plastic waste, which is one of the topmost environmental waste hazards in the world today.

**Keywords:** *Xylaria sp., biodegradation, polystyrene, plastic, scanning electron microscopy*

## Introduction

Plastic pollution has become a global ecological and societal problem, and probably one of the biggest challenges that man has ever faced. There is an estimated 3 trillion plastic waste floating in the ocean alone [1]. Polystyrene, commercially known as styrofoam, is a form of petroleum-based synthetic, thermoplastic polymer, and one of the major plastic pollutants that continue to rapidly accumulate in the biosphere by the minute contributing to what is called 'white pollution' [2,3,4]. The properties of polystyrene, namely recalcitrance, high molecular weight and hydrophobicity, make it a tough polymer to biodegrade [3,5]; however, due to its durability and low cost, it is widely

used in the industry in a variety of forms [6]. Polystyrene is one of the many solid wastes deposited in landfills and, because of its low density, it takes up a considerable amount of landfill space [7]. The inefficient management of solid waste (i.e., inadequate disposal of solid waste) have led to solid waste debris entering the ocean from land [8]. In 2013, there is an estimated 21 million tons of polystyrene used in Australia, United States and Germany alone [9].

The large-scale, ubiquitous usage and improper disposal of plastics, including polystyrene, pose a threat to a range of terrestrial and aquatic ecosystems including coastal and marine environments [4,10,11]. Plastic waste may fragment into plastic debris called microplastics (< 1 mm), and enter the food web [12]. Plastic fragments and debris in the ocean have led to the mortality of a variety of marine species (e.g., fish, invertebrates, shorebirds, marine mammals) through ingestion and/or entanglement [8,12,13]. Ingestion of plastic fragment and debris has been linked to adverse health effects [14]; accumulation of polystyrene microplastic in the gut of mice changed the microbiota composition of the gut, induced hepatic lipid disorder [15], intestinal barrier dysfunction, gut microbiota dysbiosis and bile acids metabolism disorder [16]. Plastic fragments and debris can also serve as vectors of invasive species, and carry pesticide and organic contaminants through surface absorption and adsorption spreading it to non-native and non-polluted habitats, respectively [13,17]. For example, polystyrene was found to be a source and sink of Polycyclic Aromatic Hydrocarbons, a very hazardous compound, when discarded into the marine environment [18]. Polystyrene also releases poisonous chemicals that may leak during production, disposal and incineration [17, 19]. High impact polystyrene and polystyrene is one of the most hazardous plastics based on hazard classification of monomers [19]. Styrene, the basic unit of polystyrene, is a known neurotoxin and animal carcinogen [2] and may have estrogenic effects on organisms [17,20]. The human and environmental health hazard posed by this polymer calls for urgent proper disposal methods and safe ways to breakdown this pollutant in the ecosystem.

Though polystyrene can be chemically degraded, the by-products of such process leave a noxious deposit of benzene ring, a toxic substance, in the atmosphere [2]. A safer alternative approach (i.e., without the production of harmful intermediates) is biodegradation [6]. Generally, polymer biodegradation is the process by which microorganisms (bacteria, algae and fungi), through the action of their enzymes, convert plastic waste into natural by-products such as CO2, water and minerals [21, 22]. With the facilitation of abiotic factors, biodegradation occurs in three main stages: biodeterioration, biofragmentation, and assimilation [22]. The biodegradation of polystyrene has been studied and trialed for several decades [6]. Several organisms have been reported to damage and potentially utilize this pollutant as a carbon source e.g. [3,5,23]; however, most of the literature only supports partial polystyrene degradation due to its very slow capacity to biodegrade [3,5,6,23]. Recently, the yellow mealworm Tenebrio molitor was found to feed on polystyrene as its sole carbon source; analysis of its gut revealed the presence of bacterial strains that possess the capacity to digest this polymer through enzymatic action [9]. Though this species is promising, the identification of more organisms is still of paramount importance to facilitate polystyrene elimination considering the high prevalence of this waste and its adverse environmental effects [6].

The Philippines is considered as the third highest country contributing to global mismanaged plastic waste [11]. To locally manage and find a natural and innovative solution to this worldwide concern, this study investigated the potential biodegrading ability of *Xylaria sp*. SDM (sterile dark mycelia) wild type and mutant strains on polystyrene, which is considered as one of the four most produced plastic in the world [24]. The potential ability of *Xylaria sp*. strains to utilize polystyrene as an alternative carbon source was evaluated in terms of its capacity to colonize, grow and damage the surface and subsurface structure of polystyrene using scanning electron microscopy (SEM).

## Methodology

*Study species*

*Xylaria sp*. was isolated and discovered growing on a plastic bag, buried in forest soil and litter in the lowland secondary forest Mount Makiling, Laguna, Philippines [25]. The fungus comprised of sterile melanin-pigmented mycelia and reported as ascomycete sterile dark mycelia (ASDM) [25,26]. Cultural studies have designated this fungus under Class Ascomycetes, Order Xylariales and Genus Xylaria [26]. *Xylaria sp*. was previously reported to colonize and physically damage polyethylene plastic strips suggesting it to possess the capacity to utilize the pollutant as an alternative carbon source possibly degrading it into usable form for self-sustenance [26]. The mutant strains used in the study were obtained by treatment with N-methyl-N'-nitrosoguanidine or NTG of Xylaria sp. SDM (sterile dark mycelia) wild type [27]. Protoplasts were treated with NTG and the regenerants

were characterized and scored. Mutant albino strains namely PNL 114, PNL 116 and PNL 118 were observed to have lost their melanin pigmentation and has relatively thinner hyphae compared to the wild type and the dark mutant strains namely E35 and E36.

*Preparation of Inoculum and Pollutant*

Stock cultures of *Xylaria sp.* wild type, three albino mutant strains (PNL 114, 116 and118) and two black strains (E25 and E36) were obtained from BIOTECH, University of the Philippines-Los Banos. *Xylaria sp*. strains were cultured in Potato Dextrose Agar (PDA) medium for isolation with a of pH 5.0 and incubated at 25˚C. Equal strips (1x2 cm) of clean polystyrene styro-plates purchased from the local store were weighed using a precision balance up to 0.01 g, surface sterilized by shaking in 70% ethanol (3 mins) then rinsed in sterile distilled water (1 min).

*Biodegradation using Culture Method*

Sterile polystyrene strips (1x2 cm) were placed in a sterile sealed glass jar containing 15 ml mineral medium (malt extract 1g, ammonium tartate 5g, magnesium sulphate 0.5g., calcium chloride dihydrate 0.01g, sodium chloride 0.1g, ferric chloride 0.01g, 1% w/v thiamin 5ml, 1% w/v trace elements 1ml and 1% m/v tween 80 0.2g per liter) and 0.5% glucose. The pH was adjusted to 5.0 by adding small amounts of either 0.1M sodium hydroxide or 0.1M hydrochloric acid and tested with pH paper. Two pellets of *Xylaria sp*. SDM wild type and mutant strains (PNL 114, PNL 116, PNL 118, E35 and E36) were inoculated in the sterile jars with polystyrene and mineral medium excluding the control. The jars were prepared in triplicate for each strain including the control. The fungal pellets were obtained at the margins of the colony to get actively growing and young hyphae using a cork borer with a 0.5 diameter size. The fungal strains were incubated and grown at 25ºC with a of pH 5.0 in triplicate for 50 days; optimum conditions were determined from a previous study [26]. To avoid contamination, only visual observations were undertaken on the 20th, 30th and 40th day of the incubation period. On the 50th day, the mineral medium along with the non-colonizing fungal strains were decanted from the jars, rinsed once in 70% ethyl alcohol (3 mins) with shaking, and twice with distilled water (1 min each) to remove the remaining fungi. Gentle scraping was also applied to the samples to remove the mucilaginous sheath and non-adhering fungal mycelia and on the surface. The strips were then air-dried and final weight determined using a precision balance to the nearest 0.01g.

*Determination of Initial Biodegradation through Scanning Electron Microscopy*

Since the fungal strains adhered permanently on polystyrene, percent weight loss of the pollutant was not used as a quantitative measure. As an alternative qualitative measure, samples of polystyrene were subjected to SEM to visualize fungal colonization and to assess surface damage. Samples of polystyrene per strain were subjected to SEM; factors such extent of colonization on the surface, weight increase and other physical changes were used as criteria for SEM sample consideration. Samples of polystyrene for each strain including the control were cut into small sizes (<1 cm), gold-sputtered for electrical conductivity and loaded into the scanning electron microscope (model Leica S440). Areas in the polystyrene depicting extensive growth (fungal adherence and mycelial mat formation) and surface damaged were chosen for SEM micrographs to represent *Xyalria sp*. strains' colonization on the pollutant. Surface damage on the pollutant were categorized into surface roughening and formation of cracks (line of separation), crevices (narrow openings caused by cracks) and holes (openings that larger than crevices).

## Results and Discussion

*Fungal growth and biofilm formation*

All the *Xylaria sp*. strains were observed profusely growing on the surface of all the polystyrene strips except on the control (Fig 1a-f). A thin mucilaginous sheath (biofilm) layer covered the surface of the polystyrene strips for all fungal strains throughout the incubation period except in the control set-up. Biofilm formation is regarded as the first step in microbial colonization and can be used as a qualitative indicator of a microorganism's biodegradation potential [3, 6,]. Biofilm formation functions to promote efficient substrate utilization by the microorganisms [28] enhancing their survival in environments that are low in nutrients. *Rhodococcus ruber* (strain C208), which formed biofilm on the surface and colonized polystyrene [3] and polyethylene [28], was reported to partially biodegrade the mentioned pollutants. Biofilm formation can be promoted through carbon starvation [3]; the 0.5% glucose primarily provided in the medium initiated and sustained growth for up to 3 weeks of incubation [26]. Beyond this period when glucose has been depleted, continued growth of *Xylaria sp*. SDM wild type and mutant strains can be attributed to the utilization of the alternative carbon source -i.e., polystyrene, as previously observed in *Xylaria sp*. wild type on

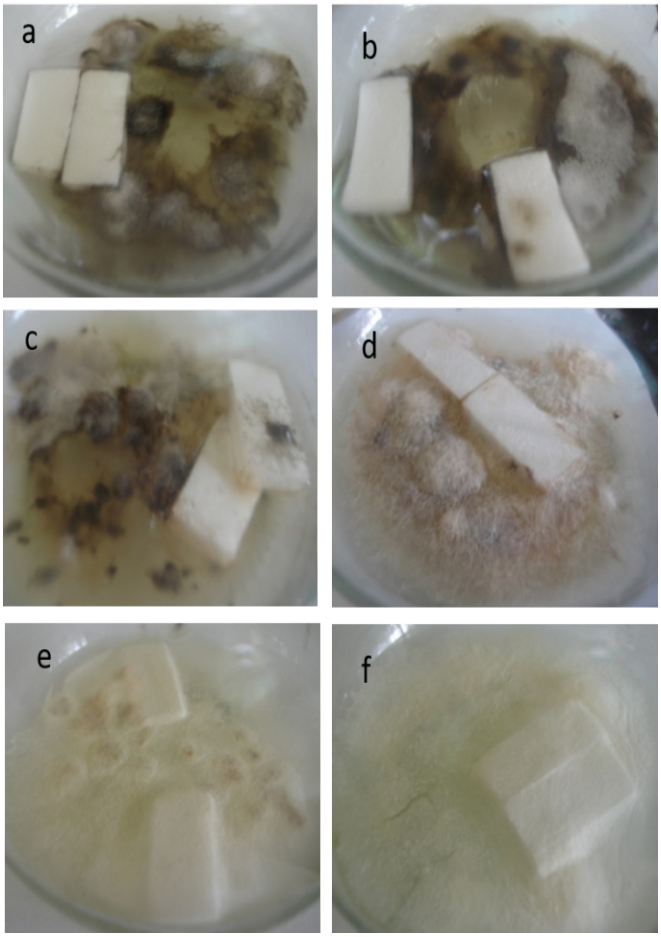


**Figure 1**. *Growth and biofilm formation of Xylaria sp. strains namely: wild type (a), PNL 114 (b), PNL 116 (c), PNL 118 (d), E26 (e) and E35 (f) in mineral medium with 0.5 % glucose observed on the 20th day of the incubation period.*

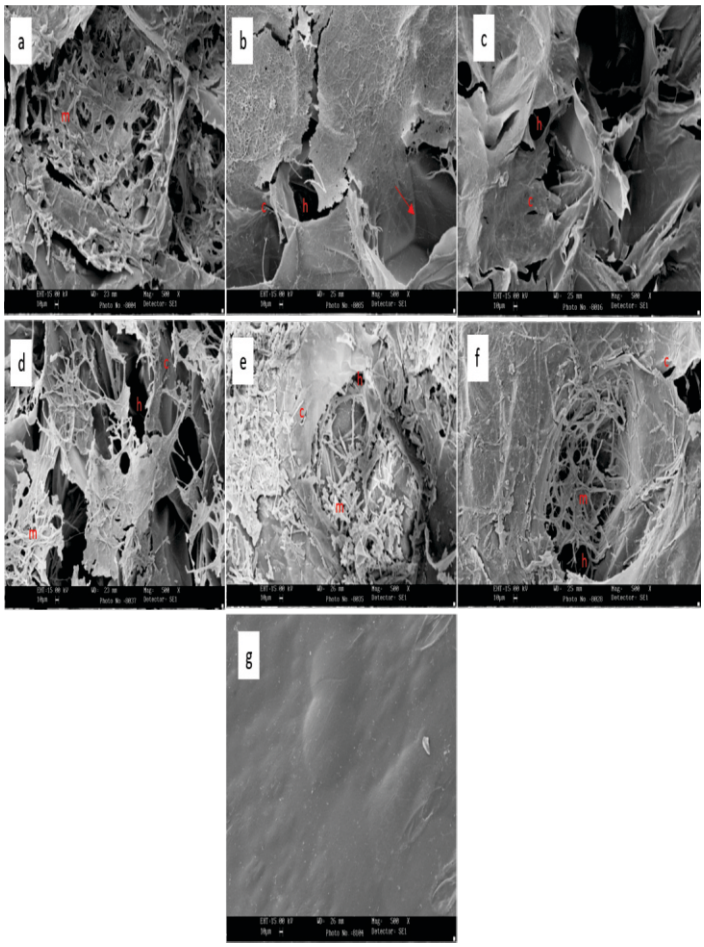


***Figure 2***. *SEM micrographs of the treated polystyrene strips inoculated with the SDM wild type (a) and the mutant strains PNL 114 (b), 116 (c), 118 (d), E26 (e) and E35 (f) showing fungal colonization, through the presence of mycelial mats (m) and hyphae, and surface damages in the form of cracks (red arrow), crevices (c) and holes (h), on the surface and sub-surface structure of the pollutant except on the control (g) (magnification at 500x).*

polyethylene plastic [26]. The capacity of *Xylaria sp*. wild type and mutant strains to utilize assimilate other carbon sources other than glucose such as polyethylene and its derivatives, PEG (polyethylene glycol) 6000, Tween 80 and tannic acid, has been previously demonstrated [27]. Hence, with the right favorable environmental conditions such as optimum pH and temperature, *Xylaria sp*. strains can thrive and grow by assimilating alternative carbon sources [26,27] apart from the pollutant used in this study.

*Fungal colonization and physical damage on polysterene*

SEM micrographs confirmed that all the Xylaria sp. strains, mutants and wild type, colonized and physically damaged the surface and inside structure of polystyrene (Fig. 2a-f) compared to the control which had no apparent damage (Fig. 2g). Physical or mechanical damages include polymer surface roughening and the creation of holes, cracks and crevices (Fig. 2a-f). Micrographs also revealed fungal adherence and penetration, through the presence of hyphae and mycelial mats, on polystyrene (Fig. 2a-f). Hyphae have apparently penetrated the pollutant and formed a profuse mycelial network on its surface and inside structure. Though all the strains grew on the pollutant, the degree of surface damage and the extent of fungal colonization cannot be quantitively assessed; hence the ability of the different fungal strains to colonize the pollutant cannot be compared significantly.

Microbial colonization is the first indication and the primary prerequisite of polymer biodegradation (23, 26). Though at this stage biodegradation through carbon utilization by Xylaria sp. strains cannot be confirmed, the study reports evidence on fungal surface adherence and penetration as well as mycelial mat embedment into the pollutant's inside structure corroborating the "active burrowing" action of *Xylaria sp.* hyphae [26]. It is suggested that microbes can adhere on the surface of the polymer by synthesizing a mucilaginous layer or a sticky layer of polymer complex [22], which was observed in the study. The mucilaginous layer enters in to the polymer material changing its structural characteristics such as porosity (size and distribution of pores), thermal transfer capacity and water or moisture content [22] allowing filamentous organisms, such as *Xylaria sp*. strains, to grow their hyphae or mycelial complex into the polymer [27]. The penetration of filamentous microorganisms, through their respective apices, facilitates polymer destruction by widening the pores or holes in the material leading to cracks [29]. It is worthy to note that gentle scraping was performed on all polystyrene strips including the control to avoid destruction of the surface and to ensure the removal off the fungus that were not adhering to the pollutant; gentle scraping of the control was performed to standardize the visualization of the results. Hence, colonization and hyphal penetration revealed in the SEM micrographs cannot be attributed to mere aggregation of mycelial mats on the polymer's surface. Similar to past literature, surface adhesion, colonization of fungus such as Curvularia sp. [22,23] and other microorganisms on polystyrene, and the subsequent destruction of the polymer surface have been reported in a number of microogranisms [3,4,22,26]. The structural damages (i.e., holes, cracks and crevices) observed in the study indicate biodeterioration or the physical destruction of the polymer's surface. Biodeterioration, which is the first stage in polymer biodegradation, occurs when microorganisms attack the surface of the polymer making the polymer chains available as carbon source to the organism [22,30,31]. A limitation of using qualitative measures (e.g., SEM, light microscope, atomic force microscopy and scanning force microscope) for visualization, is that the magnitude of polymer assimilation cannot be quantitatively measured (6). However, the use of microscopes such as light and SEM have been extensively employed in a number of biodegradation studies either as a stand-alone confirmatory method or as a complementary tool to visualize colonization and/or polymer damage [8, 23,26,28]. Moreover, there is also a possibility that *Xylaria sp.* strains have utilized additives present in the structure of the experimental polymer and that observed surface damage could also be a consequence of chemical degradation [6]. Whereas fungal growth on a polymer surface is necessary but not sufficient to conclude the process of carbon assimilation as the final biodegradation step [23], the initial colonization of *Xylaria sp*. strains on polystyrene supports its ability to establish itself and physically damage the pollutant making fungus a potential candidate organism for further polystyrene biodegradation studies.

## Conclusion

The study provides initial evidence on the ability of *Xylaria sp*. SDM wild type and mutant strains to grow and colonize polystyrene through SEM. The colonization of Xylaria sp. strains and occurrence of physical damage in the form of holes, cracks, and creves oicn the surface of polystyrene corroborates the active burrowing action of the *Xylaria sp*. strains. These results support the potentially ability of *Xylaria sp*. strains to biodegrade polystyrene plastic. The findings of this study extend the existing knowledge on the applicability of *Xylaria sp*. as a potential candidate organism to biodegrade polystyrene plastic and prospectively polyethylene plastic [26], which are among the topmost environmental waste hazards in the world today. It is recommended that further investigation and testing of the biodegrading ability of *Xylaria sp*. and its strains on other environmental pollutants be carried out, such as consideration of prolonged fungal incubation time and quantitative analyses to accurately evaluate and measure the biodegrading ability of *Xylaria sp*. strains on polystyrene and/or plastic pollutants in general. Identification of the chemical pathways involved in polystyrene biodegradation by *Xylaria sp*. including enzymes secreted in the process, by-products of biodegradation, and evidence of assimilation of polystyrene are also promising areas for future research.

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