Advanced Journal of Microbiology Research Volume 2014
Available online at http://advancedresearchjournals.org/AJMR

Full Length Research Paper

Isolation of *Fusarium* sp. AF4 from sewage sludge, with the ability to adhere the surface of polyethylene

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Accepted 2 September, 2013

Plastic materials like Polyethylene (PE) are the potential source of environmental pollution. Their presence in soil causes infertility of soil, preventing degradation of other normal substances, in addition to which it presents a danger to animal life. In the present study, a fungal strain was isolated from sewage sludge capable of adhering to the surface of PE pieces, through enrichment technique. A thick network of fungal hyphae was observed on the surface of the plastic pieces under light microscope. The fungal strain was identified as *Fusarium* sp. AF4. A visible increase in growth of the *Fusarium* sp. AF4 was observed on the surface of PE pieces, when cultured in basal salts medium at 30°C and 150 rpm, for 2 months. When the PE pieces were observed through scanning electron microscope, some changes, like appearance of pits, cracks, and erosion, were detected. CO_2 evolution as a result of PE biodegradation was calculated gravimetrically by Sturm Test. About 1.85 g/l of CO_2 was produced in case of test, whereas, 0.45 g/l in case of control. The biomass was also higher in case test as compared to control. These observations indicated that *Fusarium* sp. AF4 have the ability to degrade polyethylene.

Key words: Biodegradation, *Fusarium* sp. AF4, polyethylene (PE), Scanning electron microscopy, sturm test.

INTRODUCTION

Plastic materials are strong, light-weight, and durable and thus are widely used in food, clothing, shelter, transportation, construction, medical, and recreation industries (Orhan and Buyukgungor, 2000). More than 40 million tons of plastics produced each year (Yang et al., 2007). However, because of its xenobiotic origin and recalcitrant nature, its biodegradation is problematic and it accumulates at a rate of 25 million tons per year (Orhan and Buyukgungor, 2000).

Polyethylenes (PE) of high and low density are primarily used in product packaging as sheets and thin films. Despite of their wide applicability, the main limitation to their use is the fact that polyethylene adversely affects the environment (Satlewal et al., 2008). Polyethylene is one of the most inert plastic materials and its degradation is a very slow process, estimated in decades. Its recalcitrant nature results from its high molecular weight, complex three-dimensional structure, and hydrophobic nature, all of which interfere with its availability to microorganisms (Hadad et al., 2005).

The degradation of most synthetic plastics in nature is a very slow process that involves environmental factors and action of wild microorganisms (Albertsson, 1980; Cruz-Pinto et al., 1994; Albertsson et al., 1998). The primary mechanism for biodegradation of polymers (plastics) is the oxidation or hydrolysis by enzymes to create functional groups that improve its hydrophylicity. Consequently, the main chains of polymer are degraded resulting in polymer of low molecular weight and feeble mechanical properties, thus, making it more accessible for further microbial assimilation (Albertsson and Karlsson, 1990; Huang et al., 1990).

Biodegradation of polyethylene has been studied extensively earlier (Breslin, 1993; Breslin and Swanson, 1993) but the results were based on PE blend with starch (Pometto et al., 1993; Pometto et al., 1992). Polyethylene was claimed to be degraded but the extent could be extremely small. Other data describing degradation of PE containing starch is questionable as microbial metabolites contaminating the PE surfaces and could have been interpreted as degradation products of the parent PE (Albertsson et al., 1994). Biodegradation resulting from the utilization of polyethylene as a nutrient (that is, a

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carbon source) may be more efficient if the degrading micro-organism forms a biofilm on the polyethylene surface. However, the hydrophobicity of the polyethylene interferes with the formation of a microbial biofilm. Attempts to facilitate colonization of polyethylene by adding nonionic surfactants to the culture medium promoted the biodegradation of polyethylene (Albertsson et al. 1993; Ehara et al. 2000). Earlier reports have shown that no signs of deterioration could be observed in polyethylene sheet incubated in moist soil for 12 years (Potts, 1978), and only partial degradation was observed in a polyethylene film buried in soil for 32 years (Otake et al., 1995). Nevertheless, several studies have demonstrated partial biodegradation of polyethylene after UV irradiation (Cornell et al., 1984), thermal treatment (Huang et al., 1990; Kwpp and Jewell, 1992) or oxidation with nitric acid (Mochizuki et al., 1999).

Gilan et al., (2004) isolated a strain of *Rhodococcus ruber* that was found to colonize and degrade polyethylene. The ability of this bacterium to form a biofilm on polyethylene was attributed to the hydrophobicity of its cell surface. Addition of a small amount of mineral oil to the culture medium increased both biofilm formation and the subsequent biodegradation of the polyethylene, presumably by increasing the hydrophobic interactions between the bacterial biofilm and the polymer (Gilan et al. 2004). In another study, a thermophilic bacterial strain, *Bravibacillus borstelensis*, was isolated which was more effective in degrading branched low-density polyethylene than the *Rhodococcus* strain (Hadad et al., 2005). Watanabe et al., (2009) isolated and identified three types of low-density polyethylene (LDPE) degrading microbes *Bacillus circulans*, *Bacillus brevies*, and *Bacillus sphaericus*, by soil burial method. Different fungal strains forexample, *Mucor rouxii* NRRL 1835 and *Aspergillus flavus* (El-Shafei et al., 1998), *Penicillium simplicissimum* YK (Yamada-Onodera et al., 2001), and *Phanerochaete chrysosporium* (Iiyoshi et al., 1998), have been reported as degrading PE.

In the present study a new fungal strain *Fusarium* sp. AF4 was isolated from sewage sludge through enrichment, which was capable of adhering PE pieces. It was further used for testing their ability to use PE as a sole carbon source.

MATERIALS AND METHODS

Isolation of fungal strain colonizing the polyethylene

The sewage sludge was collected from sludge treatment plant, Islamabad, Pakistan, which was used to screen for polyethylene utilizing microorganisms. Sewage sludge (1 ml) was added to Erlenmeyer flasks containing 50 ml of the basal medium which contained polyethylene as the sole carbon source and incubated at 35^{O}C with shaking (150 rpm min^{-1}). The composition of basal medium was as follows: (g/l: K_2HPO_4 8, KH_2PO_4 1.0, $(NH_4)_2SO_4$ 0.5, $MgSO_4$ 0.2, NaCl 0.1, $FeSO_4.7H_2O$ 0.02, Na_2 MoO_4 0.0005, $MnSO_4$ 0.0005, $CuSO_4$ 0.0005, $ZnSO_4$, $CaCO_3$ 0.5, Agar 15), and pH 7. After a week, 1 ml of culture broth was transferred into the tubes containing fresh basal medium. This procedure was repeated five times. The PE pieces were washed thoroughly with sterilized distilled water, placed on glass slides and observed, microscopically (100x) using oil immersion, for any microbial growth on its surface. The rest of the pieces were shifted to the Saboraud's dextrose agar plates and incubated at 30^{O}C for 4-5 days, for isolation of fungal strain from the surface of PE.

Identification of fungal strain

Fungal strain was identified by using standard identification techniques such as colony morphology and microscopic examination. Structure of hyphae and conidiophores were observed microscopically.

Measurement of biodegradation

Scanning electron microscopy (SEM): Polyethylene (PE) plastic bags were used as the standard polyethylene for the degradation experiments. The pieces of PE (1 g) were added to an Erlenmeyer flask (250 ml) containing 100 ml of the basal medium. After inoculation with fungal strain, it was incubated at 30°C at 150 rpm for 2 months. Experiment was performed in triplicate. After incubation, the PE pieces were taken, washed with sterilized distilled water. The degradation was monitored by taking scanning electron micrographs (SEM) of microbially treated PE pieces test and compared with untreated PE pieces, taken as control.

The samples were mounted on the Aluminium stubs by silver paint. Gold coating was carried out in vacuum by evaporation in order to make the samples conducting. Microstructural examination was conducted in the Leo 440i scanning electron microscope. The images of the test samples were compared with the original untreated control samples.

CO_2 evolution test: Biodegradation of polymer chain leads to production of carbon dioxide. This CO_2 evolved as a result of PE biodegradation was determined gravimetrically by Sturm Test (Figure 1). PE pieces were added in the culture bottle (Test bottle) containing 300 ml of mineral salts medium without any other carbon source and inoculated with 1.5×10^5 spores per ml of test organism that is, *Fusarium* sp. AF4. The control bottle was without any plastic. Equal amount of air, sterilized by 0.2 filter, was allowed to flow through 3 M KOH solution containing bottles (air pretreatment chambers) which traps the CO_2 from the air. This CO_2 free air was supplied to both test and control bottles to keep conditions aerobic. The test and control bottles were stirred continuously by placing them on the magnetic stirrer. The test was performed at room temperature (30°C) for 4 weeks. After 4 weeks of culturing the difference in biomass and the amount of carbon dioxide produced in the test and control bottles was calculated. CO_2 evolved as a result of degradation of polymeric chain in the test bottle was trapped in the absorption bottles containing 1 M KOH. 0.1 M Barium chloride solution was added to both the test and the control bottles. CO_2 released during microbial respiration as well as from breakdown of polymer, absorbed in KOH containing control and test bottles, formed precipitates of barium carbonate. CO_2 produced was calculated gravimetrically by measuring the amount (weight) of CO_2 precipitates formed by the addition of $BaCl_2$. Difference in both the test and control was observed.

RESULTS

Isolation and Identification of fungal strain

The extensive network of fungal hyphae was observed on

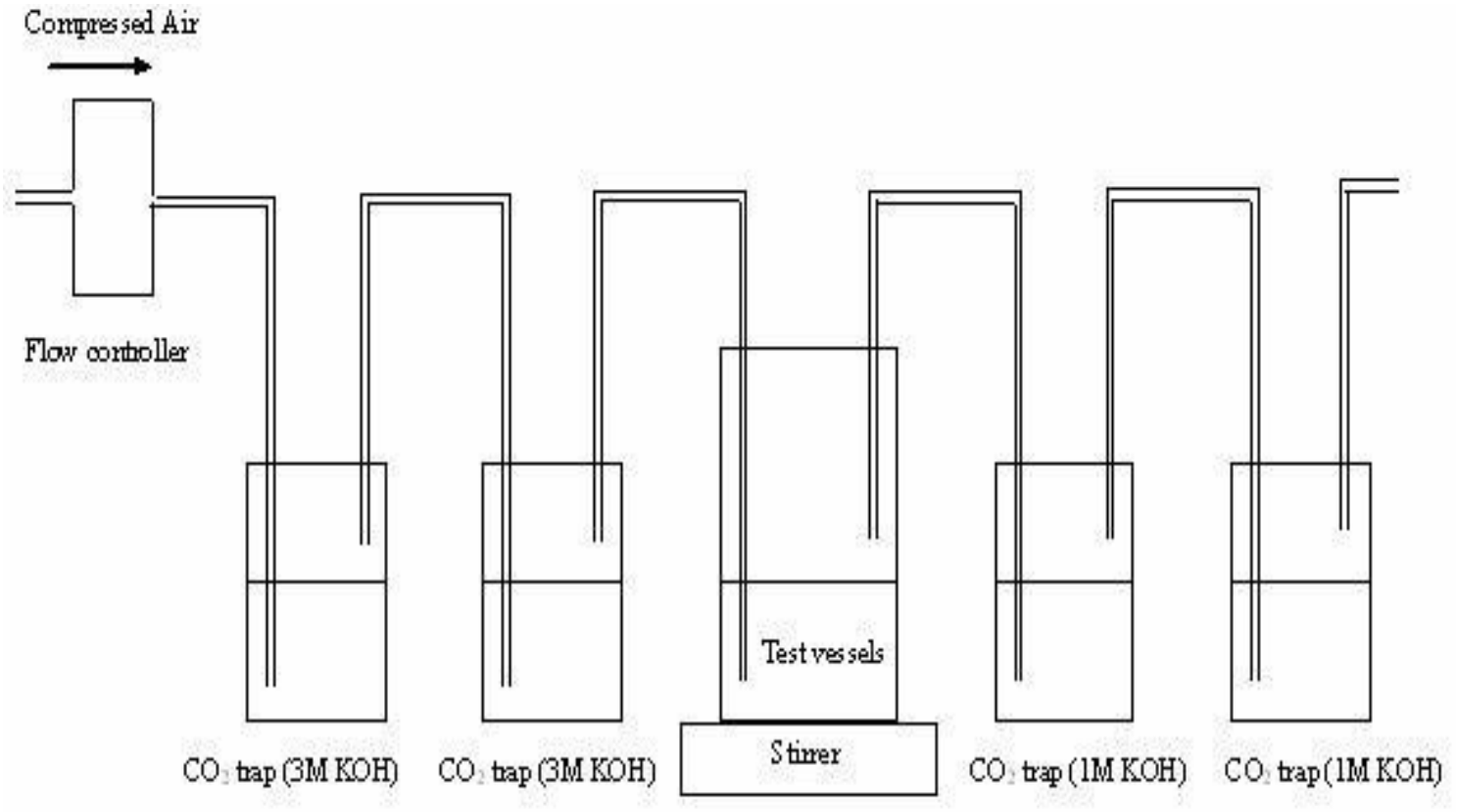

Figure 1. Schematic diagram of sturm test.

the surface of PE pieces after incubation in basal medium (enrichment experiment), under light microscope (Figure 2). On the basis of microscopic examination and morphologic characteristics, the fungal strain was identified as *Fusarium* sp. AF4. It grew rapidly on Sabouraud dextrose agar and produced woolly to cottony, flat, spreading colonies (Figure 3) . Hyaline septate hyphae and phialides, macroconidia, and microconidia were observed microscopically. Conidiophores with ovoid to elongated conidia of variable size were visible.

Measurement of biodegradation

Scanning Electron Microscopy (SEM): Scanning electron micrographs of PE pieces were taken after two months of incubation with *Fusarium* sp. AF4 in basal medium. In the test samples, there were erosions and extensive roughening of the surface with pits formation observed, whereas no such changes detected in untreated PE pieces (Figure 4).

CO_2 evolution test: The total amount of carbon dioxide evolved as calculated gravimetrically, was about 1.85 g/l in case of the test, whereas, 0.45 g/l in case of control and total increase in biomass in case of test was higher (2.281 g) than control (0.125 g) (Table 1).

DISCUSSION

In this study we have isolated a fungal strain from sewage sludge, capable adhering to the surface of polyethylene and utilizing it as a source of carbon and energy. EI-Shafei et al. (1998) investigated the ability of fungi and *Streptomyces* strains to attack degradable polyethylene consisting of disposed polyethylene bags, and isolated eight different *Streptomyces* strains and two fungi *Mucor rouxii* NRRL 1835 and *Aspergillus flavus* from sewage sludge. *Aspergillus flavus*, isolated from sanitary landfills was also found to be able to degrade PE (Mendez et al., 2007). Yamada-Onodera et al. (2001) have identified a fungus, *Penicillium simplicissimum* YK that could degrade the untreated high-density polyethylene.

We have employed enrichment technique for isolation of PE degrading microorganisms. Enrichment culture methods were also reported to be effective for isolating a thermophilic bacterium *Brevibacillus borstelensis* strain 707 capable of utilizing polyethylene as the sole carbon and energy source (Hadad et al., 2005). When polyethylene plastic pieces were examined microscopically after enrichment experiment in basal medium, fungal attachment was found on the surface of the plastic, indicating possible utilization of plastic as a source of nutrient. The

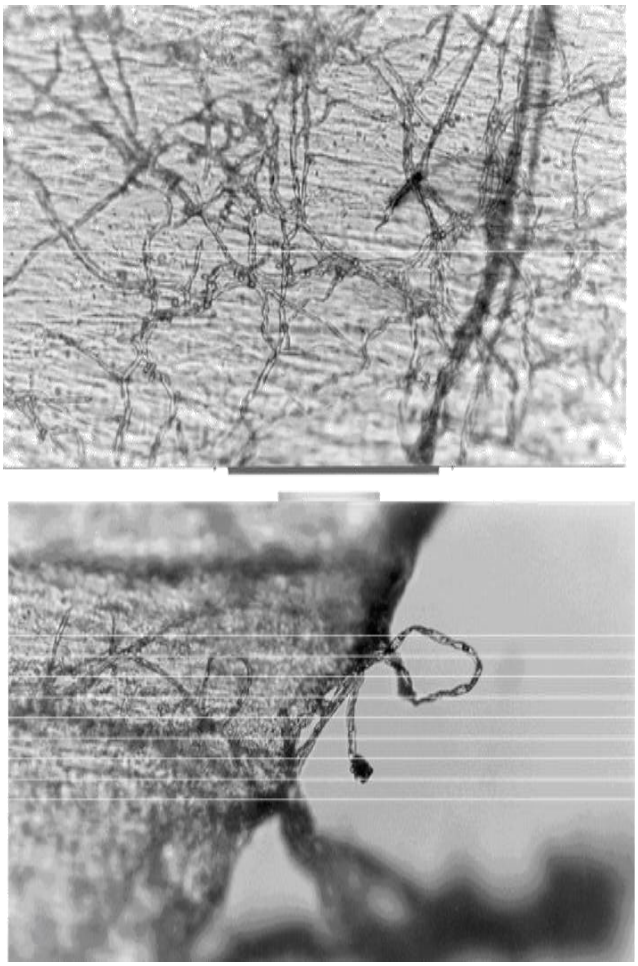

Figure 2. Microscopic examination of fungal growth on the surface of polyethylene pieces buried in sewage sludge (100x).

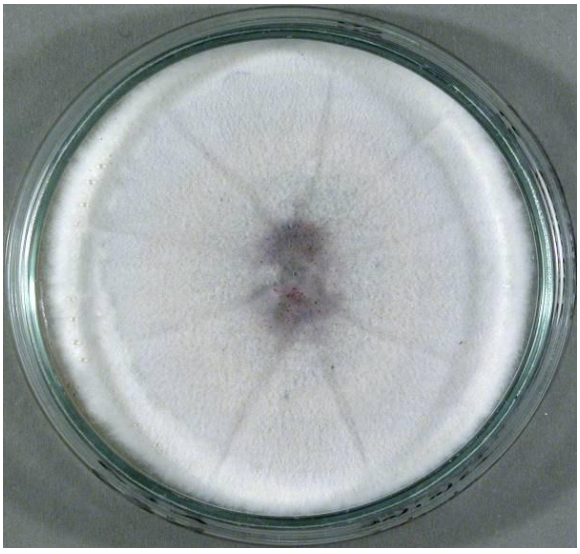

Figure 3. Morphology of colonies of fungal isolate *Fusarium* sp. AF4 on Saboraud dextrose agar plates.

ability of the fungal strains to form a biofilm on polyethylene was attributed to the gradual decrease in hydrophobicity of its surface (Gilan et al., 2004). Visible increase in growth on the surface of plastic was also observed when cultured with same isolates in basal salt medium for 2 months.

From our preliminary findings it can be concluded that sewage sludge contain fungal isolates that are capable of adhering on the surface of polyethylene films. This indicates the possibility of their ability to utilize PE as a source of nutrient leading to their biodegradation. The increase of biomass in our liquid cultures with PE confirms the ability of these microbes to utilize this polymer as a source of carbon.

The results of scanning electron microscopy indicated surface damage of PE incubated with *Fusarium* sp. AF4. Thus, it can be concluded that the fungal strains especially the *Fusarium* sp. AF4 is able to adhere to the surface of PE and can cause surface damage which can facilitate degradation of plastic.

Biodegradation can be defined as the microbial-catalyzed conversion of a polymeric substrate into biogas under aerobic (carbon dioxide [CO_2]) and anaerobic (CH_4) conditions in a biologically active environment. The Sturm test is an aerobic biodegradation technique based on CO_2 production (Calil et al., 2006). Sturm test (Sturm, 1973), manual method, is commonly employed for evaluation of the biodegradability of polymer materials (Calmon et al., 2000). Various modifications of this test have been used for the measurement of carbon dioxide evolution during degradation of polymers (Muller et al., 1992; Kim et al., 2000; Zee et al., 1998). The importance of modified Sturm tests is more in analysis of complete biodegradation of polymeric materials during biological waste processing (Kim et al., 2000). In our study we have used this technique to check the biodegradation of PE. There was a difference in the amount of carbon dioxide produced in test and control bottles indicating greater breakdown of the polymer in the test than the control.

Conclusion

In the present study, a new fungal strain *Fusarium* sp. AF4 was isolated from sewage sludge, capable of not only adhering to the surface of PE film but also utilizing it as the source of carbon and energy, as evident by the increase in growth. Although it is a slow process but studies like this gives the evidences of biodegradation of PE. It indicates that there is a great possibility of finding microorganisms from the environment which can degrade synthetic plastics.

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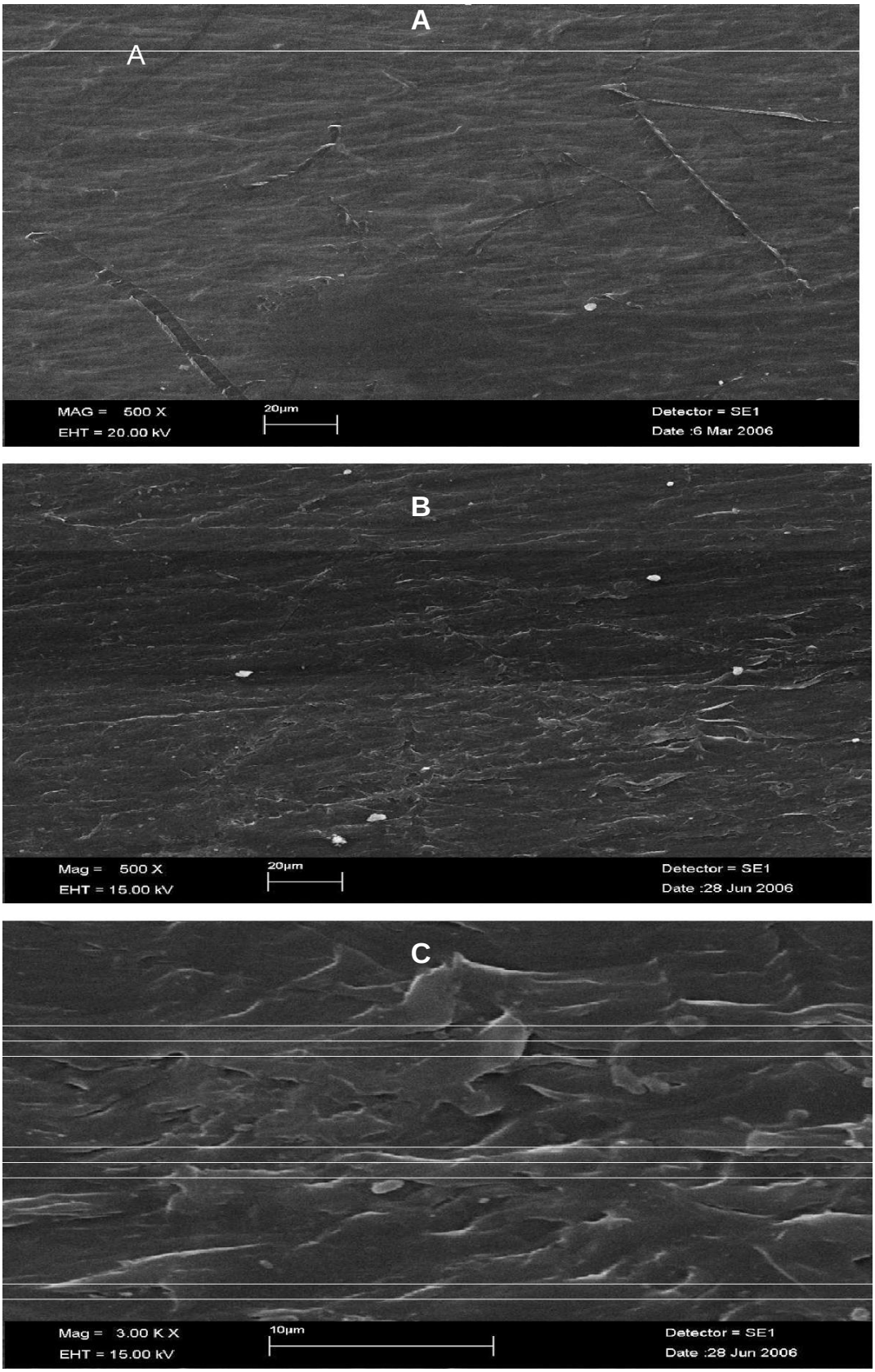

Figure 4. SEM micrographs of A) untreated PE, B) after exposure to soil burial treatment for 10 months (500x) C) magnified Fig. B (3000x).

Table 1. Gravimetric analysis of CO_2 evolution as determined through sturm test.

Sample	Total increase in biomass (g)	Amount of CO_2 produced (g/l)
Test	2.281	1.85
Control	0.125	0.45

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