International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2736-1756 Vol. 15 (1), pp. 001-009, January, 2021. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Evaluation of the biodegradability of starch based plastic polymer by some isolated composted soil fungi

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Accepted 23 November, 2020

Fourteen fungal species (*Alternaria alternata, Aspergillus candidus, Aspergillus flavus, Aspergillus niger, Aspergillus ochrochus, Botrytis cinerea, Chaetomium globosum, Fusarium moniliforme, Fusarium oxysporum, Fusarium solani, Penicillium chrysogenum, Penicillium funiculosm, Penicillium italicum* and *Phanerochaete chrysosporium*) belonging to Ascomycete, Basidiomycete and Deuteromycete groups were isolated from composted soil in Egypt. The ability of laser induced plasma as a new technique to enhance fungal degradation efficiency of starch based plastic polymer was tested. The maximum significant plastic degradation activities for all isolated fungal species were showed after the lowest exposure time (5 min) to laser induced plasma. The highest efficient fungal degraded starch based plastic polymer was *A. niger*, where the initial appearance of clear zone was recorded only after two days accompanied with the highest significant amylotic activities. The evaluation of changes in starch based plastic polymer degraded by *A. niger* compared with uninoculated and non plasma treated *A. niger* degraded starch based plastic polymer was observed by scanning electron microscope (SEM). The maximum degradation efficiency accompanied with the highest loss of tensile strength (90 and 80.7%, respectively) was observed in the plasma treated *A. niger* degrading starch polymer. Four low molecular weight sugars were detected by HPLC in plasma and non plasma treated *A. niger* degrading plastic polymer.

Key words: Fungi, plastic, degradation, laser.

INTRODUCTION

Plastic materials have become an integral part of contemporary life because of many desirable properties including durability and resistance to degradation. These plastics accumulate in the environment at a rate of more than 25 million tones per year (Konduri et al., 2011). Improper disposal of plastics has threatened natural environment worldwide since long time ago (Huey, 2006). Excessive molecular size seems to be mainly responsible for the resistance to biodegradation and their persistence in soil for a long time (Kirithika et al., 2011). Because of environmental pollution problems caused by using synthetic polymers based on petrochemicals, the development of environmental friendly polymeric materials has attracted extensive interest (Ga et al., 2005). Agro-polymers such as starch or cellulose from agro-resources have a great concern, where starch is a potentially useful material for biodegradable plastics because of its natural abundance and low cost and accordingly starch-based materials with biodegradable properties have been developed (Roldán-Carrillo et al., 2003). The interference between polymers and starch can play a critical role in obtaining composite materials with good final properties. Thermoplastic starch is obtained from native starch with the help of glycerol, water and other polyols (Mathew and Dufresne, 2002). Starch based plastics possesses the characteristic of being able to absorb humidity and is thus being used for the production of drug capsules in the pharmaceutical sector (Chua et al., 1999).

Microorganisms secrete enzymes that break down the plastic polymer into its molecular building blocks which are utilized as a carbon source for growth (Tokiwa et al., 2009). Fungi are able to degrade a wide variety of polymer (Reddy, 1995), through the production of several enzymes (cellulase, amylase) (Sethuraman et al., 1998).

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Therefore, the investigation of the ability and stimulation of fungal degrading starch-containing degradable plastics in pure culture was required.

To enhance the degradation of the plastic polymer, photo initiators are added to the microbe degradable plastic films (Anthony et al., 1993). Laser-induced breakdown (LIB), also known as laser-induced plasma (LIP) can be regarded as excitation source, since laser induced plasma can be produced in gases or liquids, as well as from conducting or non-conducting solid samples (Le Drogoff et al., 2004). LIP uses a laser pulse as the excitation source where the laser is focused to form plasma, which excites samples (Joshi, 2007). In LIP, a small volume of the target is intensely heated by the focused beam of a pulsed laser, and thus brought to a transient plasma state. LIP measurements are generally carried out in ambient air at atmospheric pressure. For this reason, and also due to its rapidity, non-contact optical nature, absence of sample preparation, very low sample consumption and excellent depth profiling, LIP consider as the preferable enhancement technique (Mohamed, 2008).

The current problems are increasing environmental pollution by plastics which have stimulated investigations to find biosynthetic materials which are also biodegradable. The ultimate goal is to have a sustainable way of disposing of plastic polymers after they've completed their life that does not harm the environment, so stimulation of fungi by plasma might be part of an environmentally friendly solution for degrading plastic waste and reduce global pollution problems.

In this paper, we evaluating the biodegradability of starch based plastic polymer by some isolated composted soil fungi. Biodegradability was evaluated by clear zone, amylase degradation ability, scanning electron microscope (SEM), weight loss and tensile strength loss of starch based plastic polymer in the presence and absence of laser induced plasma. Biological transformations of the plasma treated and non plasma treated fungal degradable plastic material compared with uninoculated one was recorded by HPLC.

MATERIALS AND METHODS

Plastic source

Starch based plastic polymer was provided by the national starch and chemical company in USA as a molded foam composed of 90% starch.

Isolation and identification of fungal species

Composted soil samples were collected from experimental farm of medicinal and aromatic plants department, National Research Center, Giza, for the study of starch-based plastic polymer degradation capabilities of the isolated fungi. The fungi were isolated on Saboraud dextrose agar medium, Czapek Dox medium and Dextrose agar medium. Samples were suspended by vortexing in sterile distilled water and allowed to stand for several minutes. The supernatants were then serial diluted. 1 ml from each dilution was plated onto the plates and incubated at 30°C for 7 days. The grown fungal colonies were counted and identified according to Moubasher (l993) and Kern and Blevins (1997).

Starch-based plastic polymer degrading ability

The basal medium contained 0.01 g yeast extract, containing about 0.02 g of dry starch-based plastic polymer as a sole carbon source and solidified with 1% agar at pH 6.0. Plastic films in culture medium were incubated with shaking for 24 h before inoculation to ensure asepsis. Culture medium was inoculated with 1 cm disc from the isolated fungal species and was incubated for 45 days at 30°C (Lee et al., 1991). Four replicates were prepared for each treatment. The degradation ability was measured by the clear zone formation. Also, the time (days) was measured for the initial appearance of clear zones (Reddy et al., 2008).

laser-induced plasma (LIP) source

The isolated fungal species were subjected to laser-induced plasma in the main laboratort in the National Institute of Laser Enhanced Science (NILES), Cairo University, Egypt. Nd:YAG Laser produces energy pulse (800 mJ) at 1064 nm at different exposure times (5, 7, 10, 15 and 20 min.).

Enhancement of fungal degradation amylotic activities by LIP

The ability of the isolated fungal species to degrade the starch-based plastic in the presence and absence of laser induced plasma at different exposure times (5, 7, 10, 15 and 20 min.) was carried out. Liquid basal medium were performed in 250 ml Erlenmeyer flasks inoculated with 1 cm disc of plasma treated and non plasma treated fungal species, with shaking at 125 rpm for 45 days at 30°C. The content of each flask was filtered through Whatman No. 1 filter paper. The filtered liquid was centrifuged at 10000 rpm for 20 min. (Roldan-Carrillo et al., 2003). Extracellular amylotic activity (clear zone, cm) was measured (Abe et al., 2006). The most efficient starch based plastic degraded fungal species was selected for further experiments.

Scanning electron microscope (SEM)

The evaluation of changes in starch-based plastic of the most efficient plastic degrading fungal species in the presence and absence of laser induced plasma were recorded. Studies were carried out by using SEM Model Phillips XL30 with accelerating Voltage 25K.V, X 420 and resolution for 50 µm (Goldstein et al., 1992).

Weight loss measurement

Plastic strips were harvested, washed in 70% ethanol to remove as much cell mass from the residual stripes as possible, dried at 45°C. The weights of each of the different films with and without plasma treated fungal species compared with the corresponding non plasma treated uninoculated one (control) were measured at different incubation periods (0 [control], 5, 15, 25, 35 and 45 days) (Witt et al., 2001). The values of degradation efficiency (DE%) obtained from weight loss method after initial (0 day) and final incubation time (45 days) were determined. DE% was calculated

using the following equation (Hosseini et al., 2010):

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

where w_0 and w_1 are the weight loss after initial and final incubation time, respectively.

Tensile strength loss determination

Plastic samples were washed in sterilized distilled water and dried at ambient temperature. The samples were cut into 12 × 2 cm (length x width). Loss in starch-based plastic tensile strength with and without plasma treated fungal species compared with the non plasma treated uninoculated one was determined at the tested incubation periods in the Faculty of Archaeology, Cairo University, Giza, Egypt, using a tensile strength testing machine (Technosat), type WPMF 250, made in the German Democratic Republic (Erlandsson et al., 1997).

HPLC analysis

Starch starch-based plastic polymer and its degradation products were determined by High-performance liquid chromatography (HPLC) (Perkin Elmer apparatus located in Microanalytical center, Faculty of Science, Cairo University, Egypt) using a stainless steel C18 reversed phase column (3.9 × 150 mm). The mobile phase was a solvent of methanol: acetontriel (9:1 v/v) at 1 ml min1. Extracts were analyzed with and without plasma treated fungal species was compared with the corresponding non plasma treated uninoculated one after 45 days incubation period.

RESULTS AND DISCUSSION

Isolation and identification of fungal species

Fourteen fungal species (*Alternaria alternata, Aspergillus candidus, Aspergillus flavus, Aspergillus niger, Aspergillus ochrochus, Botrytis cinerea, Chaetomium globosum, Fusarium moniliforme , Fusarium oxysporum , Fusarium solani , Penicillium chrysogenum, Penicillium funiculosm, Penicillium italicum* and *Phanerochaete chrysosporium*) belong to Ascomycete, Basidiomycete and Deuteromycete groups were isolated from the composted soil as shown in Table 1. *Aspergillus* followed by *Fusarium* and *Penicillium* were the leading tested genera, where the highest count were 104, 65 and 43 colonies out of 266, respectively. *A. niger* occupied the highest tested count (55 colonies), while the lowest fungal count (4 colonies) was showed with *C. globosum.* The obtained result was in accordance with Shah et al. (2008) who stated that *A. niger, P. funiculosm* and *Ph. chrysosporium* were starch plastic degrading species. This is consistent with their role in nature as organic polymer degraders. Mergaert et al. (1995) found that fungi that degrade plastic were predominantly Deuteromycetes, possibly Ascomycetes. Also Sasikala and Ramana (1996) found the highest percentage of plastic degradation among the Basidiomycete group.

Table 1. Total count of fungi isolated from the composted soil after 7 days.

Fungal species	Total count
Alternaria alternata	22
Aspergillus	104
A. candidus	12
A. flavus	30
A. niger	55
A. ochrochus	7
Botrytis cinerea	13
Chaetomium globosum.	4
Fusarium	65
F. moniliforme	20
F. oxysporum	32
F. solani	13
Penicillium	43
P. chrysogenum	6
P. funiculosm	27
P. italicum	10
Ph. chrysosporium,	15
Total count	266
Number of species	14

Starch -based plastic polymer fungal degradation ability

A total of eight isolated fungal strains (*A. alternata, A. flavus, A. niger, C. globosum, F. moniliforme, F. solani, P. funiculosm* and *P. chrysosporium*) out of the isolated fourteen fungal species showed clear zones around their inoculums sites (Table 2). The clear zones varied in clarity and initial appearance, where the maximum degrading starch based plastic ability (2 days clear zone appearance) was showed by *A. niger* , suggesting differences in metabolic states of the starch based plastic degrading isolates, diffusion rates of different enzymes in medium and the amounts and activities of the enzymes (Matavulj and Molitoris, 1992). *P. chrysosporium* was the lowest tested plastic degrading ability, where the clear zone appearance was recorded after 20 days. Lee et al. (2005) stated that some fungi grow slowly and show enzyme activity late.

Enhancement of fungal degradation amylotic activities by LIP

Laser induced plasma stimulate amylase activity, exhibiting a maximum significant activities for all isolated fungal species after the lowest exposure time (5 min), while a low amylase activities were observed after the

Table 2. Degradation ability (clear zone formation) of the isolated fungal species to starch based plastic polymer after 45 days incubation period.

Fungal species	Starch based plastic degradation ability (clear zone formation)	Initial appearance of clear zone (days) *
Alternaria alternata	+	17
Aspergillus candidus	-	-
A. flavus	+	10
A. niger	+	2
A. ochrochus	-	-
Botrytis cinerea	-	-
Chaetomium globosum.	+	12
Fusarium moniliforme	+	7
F. oxysporum	-	-
F. solani	+	10
Penicillium chrysogenum	-	-
P. funiculosm	+	15
P. italicum	-	-
Phanerochaete chrysosporium,	+	20
Number of plastic degrading species	8	

* The number of days between inoculation and the first appearance of a clear zone.

Table 3. Enhancement of fungal degradation amylotic activities (cm) by laser induced plasma at different exposure time (5, 7, 10, 15 and 20 min.).

Fungal species	Non plasma treated inoculated starch based plastic (Zero time control)	Plasma treated inoculated starch based plastic					LSD at 5%
		Exposure time (min.)					
		5	7	10	15	20	
Alternaria alternata	1.2	2.1	1.5	1.0	0.8	0.4	0.40
Aspergillus candidus	1.5	1.7	1.0	0.8	0.5	0.2	0.25
A. flavus	2.6	3.0	2.5	2.0	1.3	1.0	0.30
A. niger	5.5	7.3	6.0	4.8	4.0	3.2	0.74
A. ochrochus	2.5	3.0	2.0	1.6	1.0	0.5	0.50
Botrytis cinerea	2.0	2.2	1.7	1.0	0.7	0.2	0.21
Chaetomium globosum.	2.7	3.2	2.0	1.5	1.0	0.6	0.47
Fusarium moniliforme	3.0	4.5	3.1	2.0	1.5	1.0	0.50
F. oxysporum	2.3	3.0	2.0	1.4	0.9	0.5	0.38
F. solani	3.1	3.5	3.0	2.3	2.0	1.5	0.40
Penicillium chrysogenum	1.0	1.5	1.0	0.7	0.5	0.3	0.19
P. funiculosm	1.6	2.5	2.0	1.6	1.0	0.6	0.45
P. italicum	1.4	2.0	1.2	0.7	0.5	0.2	0.30
Phanerochaete chrysosporium	0.7	1.5	1.0	0.8	0.5	0.3	0.15
LSD at 5%	1.20	2.47	1.79	1.30	0.95	0.50	

LSD= Least significance difference.

maximum exposure time (20 min) as shown in Table 3. The radiation may stimulate the gene responsible for enzyme production (Jablonski and Chaplin, 2010). Our finding is coupled with that recorded by Kapelev (1989) who found that the exposure to laser radiation caused a 22 to 29% increase in enzyme activity. The radiation with laser for 10 min may increase the mitotic index of the cells on the third and fourth day after irradiation (Gamaeva et al., 1983). Chen et al. (2009) stated that the inhibition capability of enzymes depends on applied dose and time of irradiation. The significance of amylase production in addition to other enzymes depends on the

fact that the isolated fungal species can use starch as co-substrate for degradation, where the degradation of starch based plastics depends on the enzyme kinetic properties (Zhang et al., 1993). Singh et al. (1995) stated that radiation mutagenesis of *F. oxysporum* enhances the activity of enzymes and the hyper-mutant secretes a high level of enzymes. Kemar et al. (2003) found that higher doses of radiation are inhibitory to the growth of microorganisms. The use of different types of radiation to increase the enzyme activity of microorganisms was studied by Vladimirov et al. (2004). Laguardia et al. (2005) recorded the point mutations due to radiation which apparently enhanced the mutant to evolve more enzyme activity that degraded the precursor and accumulated enzymes.

The highest tested significant level of amylase enzymes (7.3 cm) was produced by plasma treated *A. niger* compared with non plasma treated one (5.5 cm). Kathleen et al. (2000) stated that the darkly pigmented *A. niger* (hyaline mycelium but darkly-pigmented conidia) increase their relative competitive ability under high radiation and the pigment is mainly composed of aspergilline and melanins, in particular, should increase their competitive abilities under elevated radiation. Begum et al. (2009) stated that *A. niger* was the most resistant fungal species to irradiation. *A. niger* conidia are more resistant to radiation light due to the high level radiation absorbance by their melanin pigment (Anderson et al., 2000). The highest tested efficient degraded starch based plastic polymer was *A. niger*, where the initial appearance of clear zone was recorded only after two days accompanied with the highest significant amylotic activities. Also 5 min. was the optimum stimulated tested exposure time to laser induced plasma, so the superior plasma treated *A. niger* at 5 min. exposure time will be used for the further experiments.

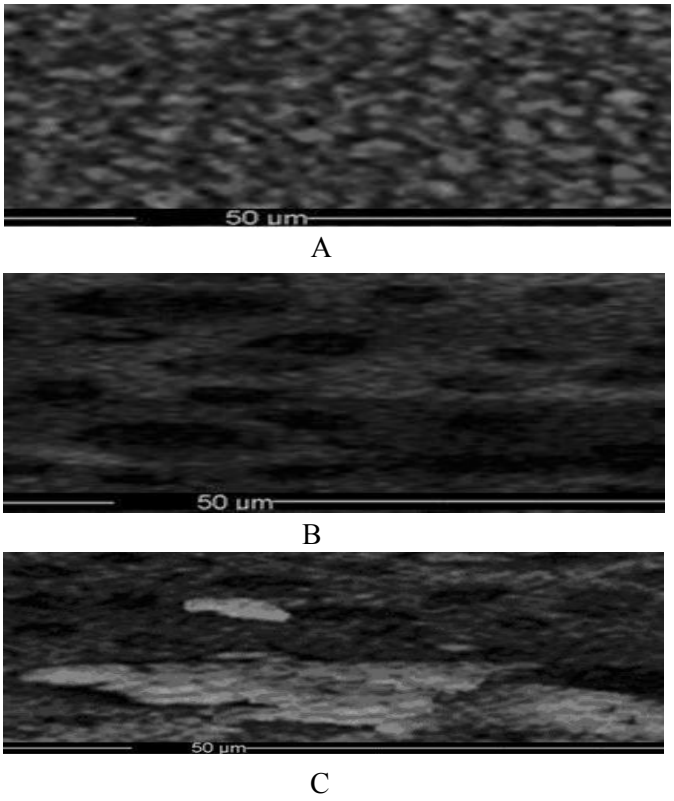

Figure 1. A- SEM of non plasma treated uninoculated starch based plastic polymer (control), B- SEM of -non plasma treated *A. niger* degraded starch based plastic polymer and C- SEM of plasma treated *A. niger* degraded starch based plastic polymer after 45 days incubation period.

Scanning electron microscope (SEM)

The evaluation of changes in the tested non plasma treated *A. niger* degrading starch based plastic polymer include formation of pits, de-fragmentation (Figure 1B), on the other hand, roughening of the surface, changes in color, formation of bio-films on the surface was showed in the plasma treated *A. niger* degrading starch based plastic polymer (Figure 1C) compared with non plasma treated uninoculated control (Figure 1A). Sang et al. (2002) reported various traces, cavities and grooves on the dented surface of plastic films demonstrating that the degradation was a concerted effect of a microbial colonizing the film surface, including fungi. Also, numerous irregular erosion pits on the surface of fungal degraded plastic have been observed by Molitoris et al. (1996). The growth of many fungi can cause small-scale swelling and bursting, as the fungi penetrate the polymer solids (Griffin, 1977). The formation of biofilms on the tested starch polymer may be explained by Gherbawy (1999) who stated that the lowest dose of radiation enhanced the growth of three isolates of *A. niger* to produce more biomass and enzymes by more than 3-fold. Also several types of low-intensity radiation were tried against different microorganisms to stimulate the growth (Karu, 2003). Radiation can penetrate the fungal cells, where it accelerates their division and protein synthesis, where the radio-mutant microbe showed a colony radial extension rate and a biomass growth rate 1.17 times higher than that achieved by the non irradiated one and the diameter of the sporangium of the mutant strains was significantly larger than that found for the parental strain (De Nicolás-Santiago et al., 2006).

Weight loss measurement

The maximum significant degradation efficiency (90%) for the plasma treated *A. niger* degrading starch polymer after 45 days incubation period compared with uninoculated and non plasma treated *A. niger* degraded plastic polymer (20 and 72%, respectively) as shown in Figure 2. The obtained data agree with Cuevas and Managilod

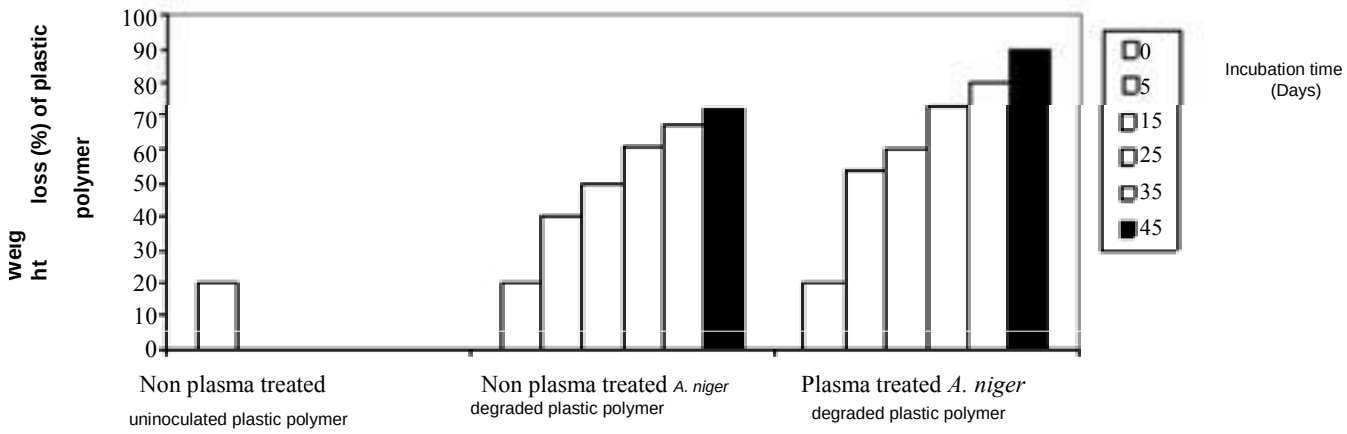

Figure 2. Effect of laser induced plasma on *A. niger* degraded starch based plastic at 5 min. exposure time on the weight loss (%) of starch based plastic polymer after different incubation period (0 (control), 5, 15, 25, 35, 45 days), LSD at 0.05= 11.20.

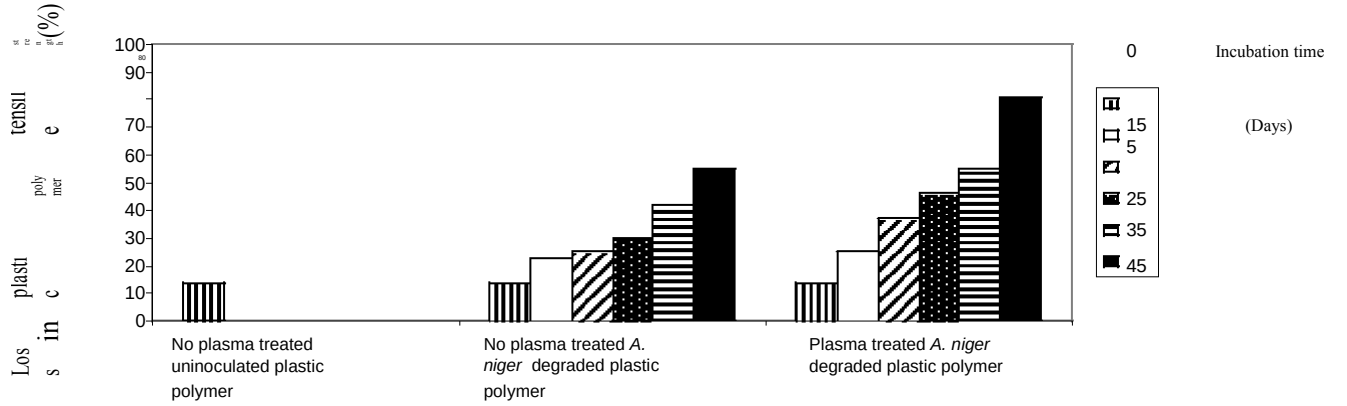

Figure 3. Effect of laser induced plasma on *A. niger* degraded starch based plastic at 5 min. exposure time on the percentage loss (%) in starch based plastic polymer tensile strength after different incubation period (0 (control), 5, 15, 25, 35, 45 days), LSD at 0.05= 6.33.

(1997) who stated that only Ascomycete mycelia developed good growth with only plastic strips as source of carbon. This isolate also caused decreases in weights of the plastic strips. Molecular degradation of polymers lead to physical and optical property changes relative to the initially specified properties involves changes to the weight of the polymer (Olayan et al., 1996). Albertsson (1980) stated that biodegradation of plastic film was reported as 0.2% weight loss. Biodegra-dation of polymer is governed by different factors that include polymer characteristics, type of organism and the nature of pretreatment, where the primary mechanism for the biodegradation of high molecular weight polymer is the oxidation or hydrolysis by enzyme. Consequently, the main chains of polymer are degraded resulting in polymer of low weight and feeble mechanical properties, thus, making it more accessible for further microbial assimilation (Huang et al., 1990). Artham and Doble (2008) stated that the polymer characteristics such as weight play an important role in its degradation. The plastic bottles exposed in aerobic soil and observed some evidences of biodegradation as reduction in weight by time (Yamada-Onodera et al., 2001). Bonhomme et al. (2003) reported that with the fungal activity, plastic with a starting molecular weight in the range of 4000 to 28,000 mg was degraded to units with a lower molecular weight of 500 mg in liquid cultivation which indicated the fungal biodegradation of that plastic.

Loss in plastic tensile strength determination

The data in Figure 3 reveal that the susceptibility of starch based plastic polymer to fungal degradation efficiency was differed significantly, where the maximum loss of tensile strength of plasma treated *A. niger* degraded starch based plastic (80.7%) after 45 days compared with uninoculated and non plasma treated *A. niger* degraded starch based plastic polymer (14 and 55%, respectively) . It means that plasma treatment of

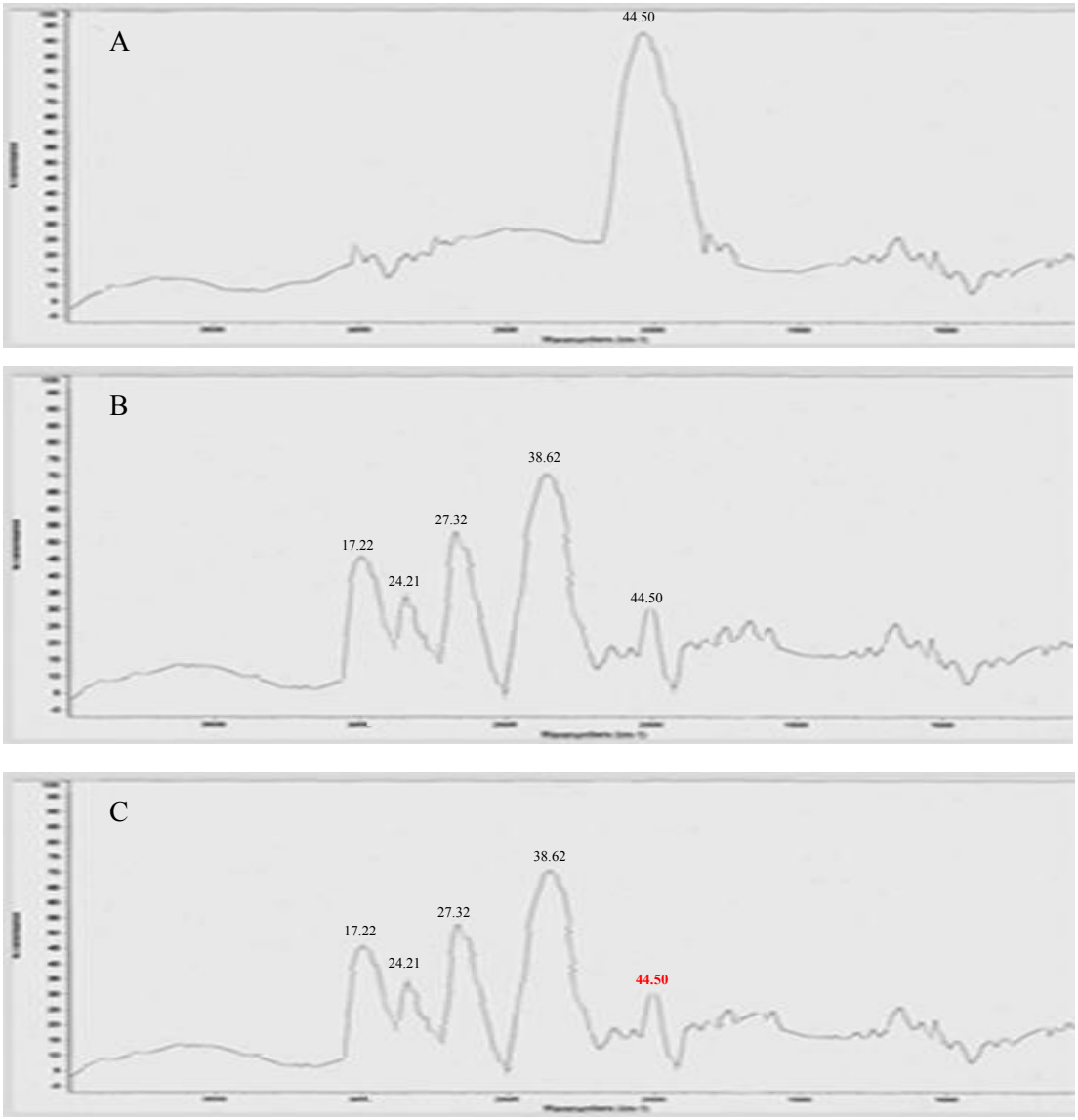

Figure 4. HPLC of starch based plastic polymer degradation products after 45 days incubation period: (A) uninoculated non plasma treated starch based plastic polymer (control), peak: 44.50-starch polymer, (B) non plasma treated *A. niger* degraded starch based plastic polymer, peaks: 46.50-starch polymer, 38.62-dextrines, 27.32-maltotriose, 24.21-maltose and 17.22-glucose (C) plasma treated *A. niger* degraded starch based plastic polymer, peaks: 44.50-starch polymer, 38.62-dextrines, 27.32-maltotriose, 24.21-maltose and 17.22-glucose.

A. niger induces loss of tensile strength on the tested starch based plastic samples. Biodegradation of plastics require modifying its mechanical properties that are responsible for plastic resistance towards degradation (Albertsson et al., 1994). This can be achieved by improving oxidation to be accessible for microbial degradation (Bikiaris et al., 1999). Tensile strength is very sensitive to changes in the molar mass of polymers, which is also often taken directly as an indicator of degradation (Erlandsson et al., 1997). Changes in physical properties such as tensile strength determining the extent of plastic biodegradation (Shah et al., 2008).

HPLC analysis

The observed result showed that non plasma treated uninoculated starch based plastic was recorded as control peak: 44.50 - starch polymer (Figure 4A) . On the other hand, four low molecular weight sugars 38.62 - dextrines, 27.32 - maltotriose, 24.21 - maltose and 17.22 - glucose) were detected in both plasma treated and non plasma treated *A. niger* degrading starch based plastic polymer by HPLC after 45 days incubation period (Figures 4B and C). The observed data reveal that starch based plastic polymer was more degraded by plasma

treated *A. niger* enzymes into more glucose units which consumed by fungus leading to more polymer degradation. Mohan and Srivastava (2010) stated that during degradation, enzymes from microorganisms break down complex polymers yielding smaller molecules of short chains (oligomers, dimers, and monomers), that are smaller enough to pass the semi-permeable outer microbial membranes, and then to be utilized as carbon and energy sources and the process is called deploy-merization. The activities of carbohydrases were shown to be enhanced after treatment with radiation, further-more, the sugar compositions of cell-wall polysaccharides were changed under the influence of radiation and carbohydrases have been shown to release oligo-saccharides (Günter et al., 2007). Amylases break the starch chains, releasing reducing sugars which consumed by the fungus (Awasuhar et al., 2000).

Conclusion

The plastic accumulate in the environment for many years and the improper disposal of it has threatened natural environment worldwide since long time ago. A study was conducted to isolate from natural environment decomposer fungi that have the capability to degrade plastic sheets. Therefore, the investigation of the enhancement ability of microbial degradation of agro-plastic polymer was required. The isolates would be used as component of new mixed fungal inocula from rapid composting of market wastes. It rather seems to be a physiological property of individual physiological strains (*A. niger*). The observed results suggest the use of laser induced plasma as a new technique to enhance *A. niger* degradation efficiency to starch based plastic polymer, where laser induced plasma technique have many advantages (the process was carried out in ambient air at atmospheric pressure, its rapidity, non-contact optical nature, absence of sample preparation, very low sample consumption, and excellent depth profiling).

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