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Microbial Colonization of High Density Polyethylene Garbage Bags

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Summary

This research aimed to determine the microbial colonization and potential biodegradation of classical high density polyethylene (HDPE) garbage bags in conditions which reflects its real disposal without preliminary abiotic pretreatment. The HDPE films were buried during 14 weeks in containers with humified soil, activated sludge and its mixture. The colonization of heterotrophic mesophilic aerobic and anaerobic bacteria on the HDPE was monitored. The bacteria and fungi formed biofilms on the surface of HDPE, but did not have the potential to degrade it since no changes in FTIR spectra of HDPE films were observed. The classical HDPE is primary nontoxic inert material, which is not biodegradable in environmental conditions, what represents a serious ecological problem.

Keywords: Bacteria, Colonization, High density polyethylene, Plastic, Resistance.

Sažetak

Cilj istraživanja bio je odrediti mikrobnu kolonizaciju i potencijalnu biorazgradnju vreća za smeće načinjenih od klasičnog polietilena visoke gustoće (HDPE) u uvjetima koji odgovaraju stvarnom odlaganju bez prethodnog abiotskog pred-tretmana. Filmovi HDPE su čuvani tijekom 14 tjedana u kontejnerima s humificiranim tlom, aktivnim muljem i njihovom mješavinom. Praćena je kolonizacija heterotrofnih mezofilnih aerobnih i anaerobnih bakterija na HDPE filmovima. Bakterije i plijesni su formirale biofilm na površini HDPE, ali nisu imale sposobnost njegova razgrađivanja, budući da nisu opažene promjene FTIR spektra HDPE filmova. Klasični HDPE je netoksični inertni materijal, koji nije biorazgradiv u okolišnim uvjetima, što predstavlja ozbiljan ekološki problem.

Ključne riječi: bakterije, kolonizacija, polietilen visoke gustoće, plastika, otpornost.

1. Introduction

Polyethylene is a polymer of long chains of the monomer ethylene. It is one of the world's most common plastics, with a wide range of uses and over 60 million tons produced worldwide every year. Based on the material density and branching, the common types of polyethylene are known as high density, medium density and low density polyethylene. High density polyethylene (HDPE) is little branching linear thermoplastic with density greater or equal to 0.941 g cm^{-3}. HDPE has a wide variety of applications and the global marked reached a mass of more than 30 million tons in 2007 (Ceresana research, 2008).

When the products from polyethylene reach the end of their use, most are discarded directly in landfills without any previous treatment. The recycling of polyethylene is not being used extensively due to lack of suitable infrastructure for sorting, processing and composting. Biodegradation of polyethylene implies the decomposition of material into carbon dioxide, methane, water, inorganic compounds and microbial biomass, in a specific period of time, reflecting the available disposal conditions. The methods for studying the biodegradation of polyethylene use the burial of material in soil, compost, sewage sludge or pure microbial cultures in laboratory conditions (Singh and Sharma, 2008).

The classical commercial non-oxidized polyethylene is not biodegradable and is one of the most inert synthetic polymers. Polyethylene buried in soil during 10 years evolved less than 0.2% of carbon dioxide produced (Albertsson and Karlsson, 1990). Polyethylene films buried in soil during 32 years showed only partial degradation (Otake et al., 1995). Biodegradation of polyethylene has been studied extensively, but all successful results were observed only for polyethylene containing additives such as starch and commercial prooxidants. The starch contained in polyethylene is preferentially removed by microbial activity leaving the polyethylene network (Orhan et al., 2004). Polyethylene formulated with transition metal prooxidants (notably iron complexes), after ageing or weathering by exposure to UV wavelength around 300 nm, support microbial growth (Bonhomme et al., 2003; Singh and Sharma, 2008). In this case the biodegradation is evident as utilization of the carbonyl residues formed in the photooxidized polyethylene (Gilan et al., 2004). It is proposed that biodegradation of polyethylene is a two step process involving an initial abiotic oxidation, followed by microbial cleavage of the polymer carbon backbone (Gu, 2003). The environmental friendly polymer materials are available since 1990. However, these biodegradable polymers are usually more expensive than classical polymers and also have relatively slow degradation rate, which result in the extensive use of classical polymer in human everyday use.

Presently, very little is known about the biodegradation of synthetic polymeric materials such as HDPE. Since the main chains of HDPE have no accessible hydrolysable groups, it is not capable of self-decomposition and has relatively slow rate of degradation in natural environments (Gu, 2003). The degradation of HDPE in natural environments is estimated in decades and posses serious environmental concerns. Moreover, the polyolefins with molecular weight higher than 500 – 620

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g mol^{-1} have been considered resistant to biodegradation (Bonhomme et al., 2003; Singh and Sharma, 2008) and in the case of important polymers, such as HDPE, the minimum molecular weight number at about 25.000 g mol^{-1}. HDPE could be a potential source of carbon for heterotrophic microorganisms such as bacteria and fungi. Microbial degradation of water non-soluble HDPE requires the formation of a biofilm on its surface to enable microorganisms to utilize the HDPE as substrate. Microbial degradation of HDPE will depend on the presence of specific microorganisms on its surface and environmental condition (temperature, moisture, dissolved oxygen) where the biodegradation takes place. Biodegradation of HDPE was rarely considered in quantitative matter since the presence of microorganisms on the surface of plastic has generally been assumed as biodegradation. Actually, the interpretations of these data deal with the potential of biodegradation and not actually biodegradation.

The aim of this study was to determine the potential biodegradation of classical HDPE garbage bags in conditions which reflects its real disposal without preliminary abiotic pretreatment. The microbial potential for biodegradation was measured as the number of immobilized microorganisms on the surface of HDPE, while chemical changes were measured by Fourier transforms infrared (FTIR) spectra.

2. Experimental

2.1. Experimental procedure

A black high density polyethylene (HDPE) garbage bag of thickness 30 μm was obtained from Croatian packaging producer MI-PLAST d.o.o., Rijeka. Garbage bag was cut into 3 x 5 cm pieces and the HDPE films were autoclaved at 112^{0}C for 90 min. Eight pieces of sterile HDPE films were kept in sterile Petri dish, while other 24 pieces were tested for microbial degradation. The experiments were designed as the soil burial method (Orhan et al., 2004) with humified soil and settled (30 min in Imhoff cone) activated sludge from a municipal wastewater treatment plant as the substrates for microbial degradation. The humidity of substrates was adjusted to water content of 70-80% during whole experiment. A wet substrate was filled in 2L plastic containers and plastic films were deposed at 10-15 cm depth. First container contained the humified soil (H), second contained the settled activated sludge (AS) and third contained mixture of one half of humified soil and one half of settled activated sludge (HAS) as substrate. Experiments were carried out for 14 weeks in a dark at 22±0.1^{0}C.

2.2. Analytical methods

The number of viable bacterial cells immobilized onto plastic films was determined as colony-forming units (CFU) after 1, 3, 6 and 14 weeks of incubation. Two plastic films were aseptically taken each time from the container. Films were washed three times with 100 mL of sterile 0.3% NaCl solution, and aseptically placed into a tube containing 9 mL of 0.3% NaCl. The sample was mixed with a sterile glass rod and vigorously shaken on a mechanical shaker (40 Hz/5 min, Kartell TK3S). This procedure (Hrenovic et al., 2009) detaches immobilized cells from the carrier so that they remain as planktonic cells in the suspension. From such suspension serial dilutions (10^{-1} to 10^{-8}) were prepared. Volumes of 0.1 mL were then aseptically inoculated onto nutrient agar plates (Biolife) by spread-plate method. One series of inoculated plates was directly incubated in thermostat in order to obtain the number of aerobically frown bacteria. Another series of inoculated plates was incubated in Anaerocult A, Merck in order to obtain the number of anaerobically grown bacteria. After the incubation (22±0.1°C/5 days) the bacterial colonies were counted and the remaining films were dried (45°C/24 h), equilibrated to ambient temperature and weight. The number of bacteria was reported as immobilized CFUs per one gram of the film. Measurements were done in duplicate, with mean values presented. The number of immobilized fungi was determined on the same plates as bacteria.

As many as visually distinguishable different colony types of bacteria were isolated on nutrient agar plates. Morphologically different colonies were picked up by a sterile inoculation needle and grown in nutrient agar slants for further characterization. Pure cultures of bacteria were stained by Gram method and examined in a light microscope (Olympus, CX21) under the immersion objective at the magnification of 1000 x. The catalase activity was determined in 3% H_2O_2. The cytochrome oxidase activity was determined using the Oxiboswab, Biolife. The activity of the hydrolytic enzyme gelatinase of bacterial isolates was determined on nutrient agar with the addition of 1.5% of gelatine (Kumar et al., 2007).

The chemical characteristics of plastic films were evaluated at the end of experiment. Plastic films which were used for the determination of immobilized bacteria were dried at 45°C for 24 h and equilibrated to ambient temperature. The FTIR spectra of samples were recorded on a Bomem MB 100 mid FTIR spectrophotometer. Plastic films were affixed directly to standard infrared sample plates. The weight loss of each sample was calculated from the difference of mass of dry films (45°C for 24 h) measured before and after 14 weeks of experiment.

2.3. Statistical analyses

Statistical analyses were carried out using Statistica Software 8.0 (StatSoft, Tulsa, USA). The numbers of CFU were logarithmically transformed beforehand to normalize distribution and to equalize variances of the measured parameters. The comparisons between samples were done using the one-way analysis of variance (ANOVA) and subsequently the post-hoc Duncan test was performed for the calculations concerning pair-wise comparisons. The correlation between variables was estimated by Spearman correlation analysis. Statistical decisions were made at a significance level of $p < 0.05$.

3. Results and Discussion

Microbial degradation of hydrophobic HDPE requires the formation of a biofilm on its surface to enable microorganisms to utilize the HDPE as a source of carbon. Therefore, the methods of indirect measurements of microbial activity such as carbon dioxide production or the number of microorganisms in substrate used for biodegradation studies have some disadvantages. The measurement of carbon dioxide production during microbial degradation of polyethylene buried in soil, compost or sewage sludge when compared to the blank substrate (Albertsson and Karlsson, 1990; Orhan et al., 2004)

can be the consequence of nonhomogenic natural substrate and nonhomogenic distribution of microorganisms in substrate. The higher number of naturally present microorganisms in substrate will develop more carbon dioxide from substrate which has more organic compounds available for microbial metabolism. Measurement of number of microorganisms present in substrate used for biodegradation (Kumar et al., 2007; Orhan et al., 2004) does not have to represent the number of microorganisms which have the potential to degrade HDPE. Namely, the microorganisms can be present in high numbers in substrate, but never have to come in contact with HDPE. Thus the methods for determination of biofilm (Gilan et al., 2004; Hrenovic et al., 2009) formed on the surface of HDPE films seems to be the most relevant to find microorganisms capable for its degradation. Both aerobic and strictly anaerobic microorganisms are involved in degradation of polymers (Gu, 2003), thus having the potential to degrade the HDPE films. Although the experimental reactors were held aerobically, the local anaerobic conditions in substrate most probably occurred. Therefore, the number of both aerobic and anaerobic microorganisms was monitored during the experiment.

The numbers of aerobically and anaerobically incubated bacteria which were immobilized onto HDPE films are presented in Table 1. The immobilization of bacteria onto HDPE films was observed after only one week of contact with H, AS and HAS. The numbers of immobilized bacteria increased with time and the biofilm formation on HDPE films buried in H, AS and HAS showed significantly positive correlation (R = 0.944-0.998). The numbers of aerobically grown bacteria were higher than the numbers of anaerobically grown bacteria. The observed numbers of immobilized aerobically grown bacteria after 14 weeks of incubation (4-63 x 10^5 CFU g^{-1}) were comparable to the 3.3 x 10^5 - 1.11 x 10^{11} CFU g^{-1} reported for HDPE films incubated for eight weeks in mangrove soil (Kumar et al., 2007). The numbers of immobilized bacteria after 14 weeks of experiment were the highest (p<0.05) on HDPE films aged in H, followed by those aged in HAS and AS. The incubation time of 14 weeks should be sufficient to observe any biodegradation. Orhan et al. (2004) reached maximum bacterial growth at six weeks in soils with LDPE, HDPE and biodegradable polyethylene. The maximum weight loss of the materials was

reached after 17 weeks and no significant additional loss was observed until 36 weeks. Kumar et al. (2007) observed bacterial colonization and biodegradation of commercial polyethylene bags in mangrove soil after 8 weeks.

The numbers of aerobically and anaerobically grown fungi which were immobilized onto HDPE films are presented in Table 2. The first immobilization of fungi on HDPE films was observed after three weeks of incubation in H, AS and HAS. The numbers of immobilized fungi increased up to 14 weeks of experiment and the biofilm formation on HDPE films buried in H, AS and HAS showed significantly positive correlation (R = 0.972-0.998). Opposite to growth of bacteria, the numbers of anaerobically grown fungi were higher than those grown aerobically. The numbers of fungi were notably lower than the numbers of bacteria during whole period of incubation. The observed numbers of immobilized aerobically grown fungi after 14 weeks of incubation (1-19 x 10^3 CFU g^{-1}) were something higher than the 0.3-6 x 10^3 CFU g^{-1} reported for HDPE films incubated for eight weeks in mangrove soil (Kumar et al., 2007). As in the case of bacterial numbers, the numbers of immobilized fungi after 14 weeks of experiment where the highest (p<0.05) on HDPE films aged in H, followed by those aged in HAS and AS. These suggest that the humified soil was the most suitable substrate which contained the highest numbers of microorganisms capable for the potential biodegradation of HDPE films.

Table 2. *Number of aerobically and anaerobically incubated fungi immobilized per dry mass of HDPE films during 14 weeks of experiment. The control sample stayed sterile. H-sample aged in humified soil, AS-sample aged in activated sludge, HAS-sample aged in mixture of humified soil and activated sludge. 1g of HDPE film equals to 880 cm².*

Week	H	AS	HAS
Aerobic fungi (10^3 CFU g^{-1})			
1	0.0	0.0	0.0
3	0.2	0.2	0.2
6	4.6	0.8	1.2
14	18.8	1.3	6.1
Anaerobic fungi (10^3 CFU g^{-1})			
1	0.0	0.0	0.0
3	0.4	0.2	0.3
6	7.9	0.9	5.7
14	35.4	3.9	10.6

Characterization of bacterial strains immobilized on HDPE films (Table 3) showed a greater diversity among aerobically cultivated bacteria (25 isolates) than those cultivated anaerobically (10 isolates). The total number of isolates did not differ significantly among reactors containing H, AS or HAS. Most of the isolates were Gram positive (69%) rod shaped bacteria (77%). Spore formation and the presence of capsule was not a dominant future of the isolates (31%). The majority of the isolates were catalase positive (86%) and oxidase negative (69%). At least two categories of microbial enzymes are actively involved in biological degradation of polymers: extracellular and intracellular depolymerases (Gu, 2003). The enzymes which catalyze degradation of HDPE to yield a measurable product have still not been identified. In literature (Kumar et al., 2007),

Table 1. *Number of aerobically and anaerobically incubated bacteria immobilized per dry mass of HDPE films during 14 weeks of experiment. The control sample stayed sterile. H-sample aged in humified soil, AS-sample aged in activated sludge, HAS-sample aged in mixture of humified soil and activated sludge. 1g of HDPE film equals to 880 cm².*

Week	H	AS	HAS
Aerobic bacteria (10^4 CFU g^{-1})			
1	0.5	0.5	0.9
3	1.4	4.6	13.0
6	16.4	9.1	34.2
14	634.4	40.3	175.8
Anaerobic bacteria (10^4 CFU g^{-1})			
1	0.3	0.2	0.1
3	1.3	1.1	3.4
6	20.7	1.3	13.6
14	418.8	10.9	61.4

Table 3. *Characteristics of aerobically and anaerobically incubated bacterial strains immobilized onto HDPE films after 14 weeks of experiment. H-sample aged in humified soil, AS-sample aged in activated sludge, HAS-sample aged in mixture of humified soil and activated sludge.*

Reactor/ isolate	Shape	Gram stain	Spore formation	Capsule	Catalase	Oxidase	Gelatinase
Aerobic bacteria							
H1	rod	-	-	-	+	+	-
H2	rod	-	-	-	+	+	-
H3	rod	+	+	-	+	-	-
H4	rod	+	-	+	+	-	-
H5	rod	+	-	+	+	+	-
H6	filament	+	-	-	+	+	-
H7	rod	+	-	-	+	-	+
H8	filament	+	-	-	+	-	+
AS1	rod	+	+	-	+	-	-
AS2	rod	+	+	+	+	-	-
AS3	rod	+	+	+	+	-	+
AS4	rod	+	-	+	+	+	-
AS5	rod	-	-	+	+	+	+
AS6	rod	+	+	-	+	-	-
AS7	rod	-	-	+	+	+	+
HAS1	rod	-	-	-	+	-	+
HAS2	rod	-	-	-	+	+	-
HAS3	rod	+	-	+	+	-	-
HAS4	rod	+	-	+	+	-	-
HAS5	rod	+	+	-	+	-	-
HAS6	filament	+	-	-	+	-	-
HAS7	rod	+	+	-	+	+	-
HAS8	rod	-	-	-	+	+	-
HAS9	filament	+	-	-	+	-	-
HAS10	filament	+	-	-	+	+	-
Anaerobic bacteria							
H9	rod	+	+	+	-	-	-
H10	filament	+	-	-	-	-	-
H11	filament	+	-	-	+	-	-
AS8	rod	-	-	-	-	-	+
AS9	rod	+	+	-	+	-	+
AS10	rod	+	+	+	-	-	+
AS11	rod	-	-	-	-	-	+
HAS11	filament	+	-	-	+	-	-
HAS12	rod	-	-	-	+	-	+
HAS13	rod	-	+	-	+	-	-

the gelatinase activity is often used to assume the potential of polymer degradation. In our study a 35 morphologically different bacteria were isolated from HDPE samples and only 31% of isolates exhibited the gelatinase activity. The diversity of fungal species was much lower than diversity of bacteria. Only two fungal species were isolated during whole study.

The FTIR spectra of HDPE films aged for 14 weeks in H, AS and HAS showed no significant changes when compared to the control sample (Fig. 1). The C-H and C-C absorption bands of methylene group in polyethylene (2919-2851, 1463-1436 and 730-719 cm^{-1}) appeared within the same range and at the same intensity as in the spectra of control sample. Also no appearance of some new absorption bands was perceived. This suggests that although a relatively high biofilm formation was observed on HDPE films, the immobilized bacteria and fungi did not degrade the polymer carbon backbone. The weight loss of HDPE films after 14 weeks of experiment was negligible and averaged 1.07, 0.71 and 0.36% for H, AS and HAS samples, respectively. These results are comparable to 0.26% of weight loss of HDPE films buried for 13 weeks in soil mixed with municipal solid waste compost (Orhan et al., 2004). The low weight loss of HDPE films is not supported by the changes in FTIR spectra. These suggest that negligible degradation of HDPE films probable occurred only at the edges of films, which were not analysed by FTIR analysis.

In this study it was shown that the natural microbial population present in H, AS and HAS have a high potential to colonize the surface of HDPE films, but do not have the potential
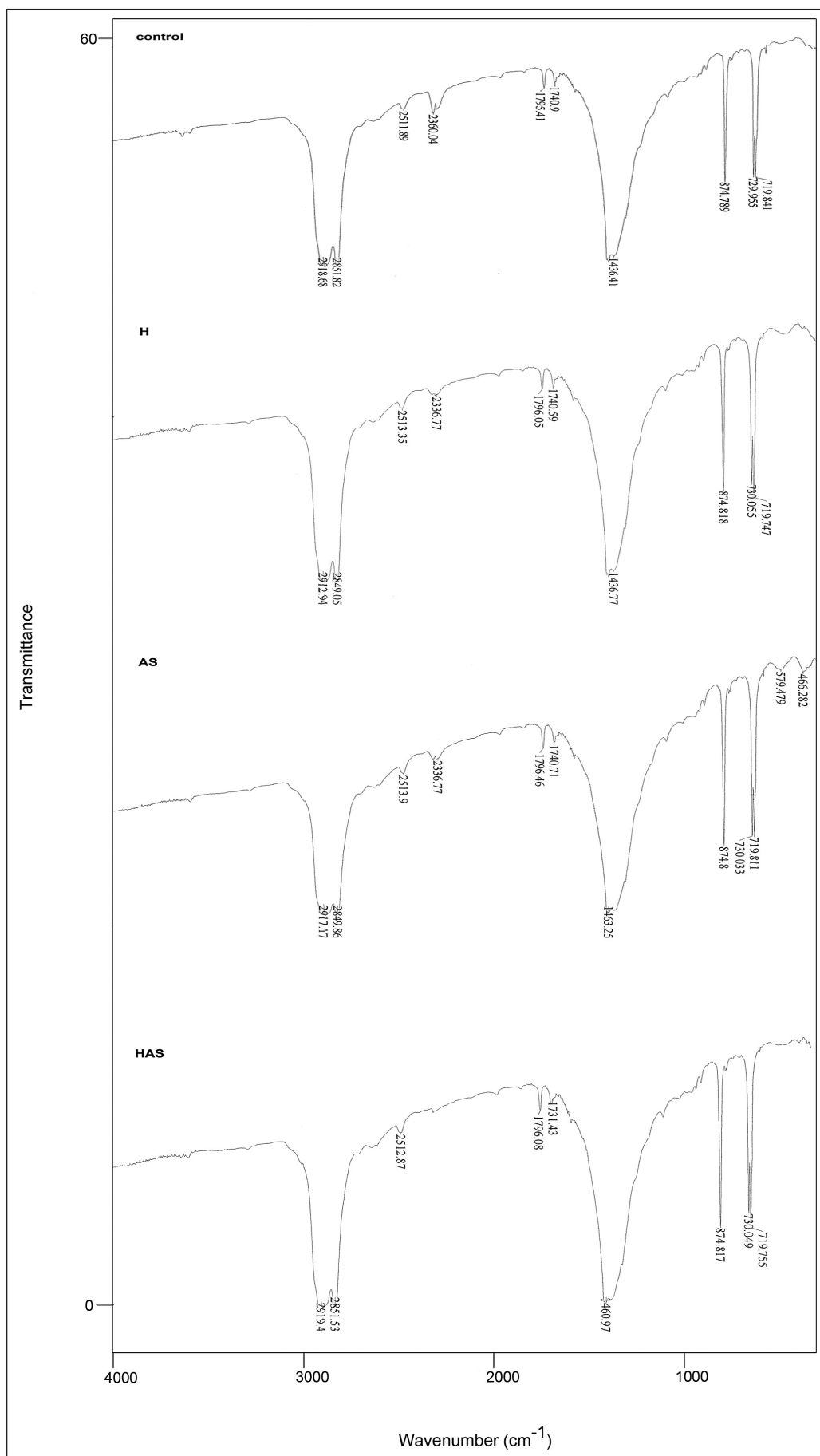
Figure 1. FTIR spectra of HDPE films after 14 weeks of experiment. Control-aseptically aged sample, H-sample aged in humified soil, AS-sample aged in activated sludge, HAS-sample aged in mixture of humified soil and activated sludge.

to degrade it. The bacterial colonization of HDPE can be due to the presence of fimbriae and extracellular substances on the cell surface. The result of unsuccessful biodegradation of classical HDPE films was not surprising according to the literature data on this topic. Namely, the unchanged infrared spectrum was also reported for HDPE films buried for 60 weeks in mixture of soil and compost (Orhan et al., 2004). The negligible degradation was reported for HDPE films buried in soil during 10 years (Albertsson and Karlsson, 1990) or even during 32 years (Otake et al., 1995). Moreover, no signs of biodegradation were found for low density polyethylene containing 12% of starch or 30% of polycaprolactone during five months of incubation in activated sludge (Gilmore et al., 1993). In polyethylene containing 9% of starch and commercial prooxidant the starch was preferentially removed by microbial activity leaving the polyethylene network (Orhan et al., 2004).

The poor biodegradation of HDPE films in soil, compost or sewage sludge can be explained from the ecological point of view. The microorganism present in natural substrate will firstly use all organic compounds present in substrate, before the use of synthetic HDPE, which is recognised by microorganisms as xenobiotic. Unfortunately, these conditions of biodegradation reflect the real conditions of disposal of HDPE. The biodegradation of HDPE in mineral salt medium (Gilan et al., 2004; Kiatkamjornwong et al., 1999) will surely give better results. In these conditions microorganisms will be forced to use the HDPE as a sole source of carbon.

It can be summarised that the HDPE is not primary toxic compound, but it's not biodegradable, which posses the serious environmental problem due to the fact that its degradation rate is slower than the accumulation rate. The sustainable development on this problem can be achieved through the recycling of waste HDPE and using the environmental friendly polymer or alternative materials.

4. Conclusion

The natural microbial population present in H, AS and HAS had a high potential to colonize the surface of HDPE films. The immobilized bacteria and fungi were not efficient in biodegradation of HDPE, as indicated by unchanged FTIR spectra of treated HDPE films. The classical HDPE is primary nontoxic inert material, which is not biodegradable in environmental conditions, which represents a serious ecological problem.

Acknowledgements

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