Bioremediation of MP-polluted Waters Using Bacteria *Bacillus licheniformis*, *Lysinibacillus massiliensis*, and Mixed Culture of *Bacillus* sp. and *Delftia acidovorans*

D. Kučić Grgić,[a,*] M. Miloloža,[a] E. Lovrinčić,[a] A. Kovačević,[a] M. Cvetnić,[b] V. Ocelić Bulatović,[c] V. Prevarić,[b] K. Bule,[b] Š. Ukić,[b] M. Markić,[d] and T. Bolanča[b,e]

[a]Department of Industrial Ecology, Faculty of Chemical Engineering and Technology, University of Zagreb, 10 000 Zagreb, Croatia

[b]Department of Analytical Chemistry, Faculty of Chemical Engineering and Technology, University of Zagreb, 10 000 Zagreb, Croatia

[c]Laboratory for Industrial Ecology, Faculty of Metallurgy, University of Zagreb, 44 000 Sisak, Croatia

[d]Department of Measurements and Process Control, Faculty of Chemical Engineering and Technology, University of Zagreb, 10 000 Zagreb, Croatia

[e]University North, 48 000, Koprivnica, Croatia

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Microplastic particles (MPs) are widely distributed pollutants in the environment. While a growing number of studies have shown that MPs are toxic to plant and animal life, systemic efforts to reduce their presence have been scarce. Low-density polyethylene (LDPE) and polystyrene (PS) are one of the most common among all plastic-forming MPs. In this study, pure bacterial strains, *Bacillus licheniformis* and *Lysinibacillus massiliensis*, and a mixed bacterial culture of *Delftia acidovorans* and *Bacillus* sp., were used for biodegradation of LDPE and PS microplastics. Biodegradation of MP-PS and MP-LDPE of particle size 300 – 500 μm was carried out under batch operating conditions at a temperature of 25 ± 2 °C, pH values of 7.15, and 160 rpm during 22 days. The obtained results showed that mixed bacterial cultures degraded MP-LDPE and MP-PS better than pure bacterial cultures, and the biodegradation efficiency was higher for MP-LDPE than for MP-PS, as indicated by greater reduction in peak intensity and spectral deformation, higher colony forming unit (CFU), and inorganic carbon (IC) values.

Keywords:

bioremediation, microplastics, LDPE, PS, *Bacillus licheniformis*, *Lysinibacillus massiliensis*, *Delftia acidovorans*, *Bacillus* sp.

Introduction

Plastics are synthetic polymer materials that are among the most frequently used materials due to their versatility, durability, light weight, chemically inert behaviour, and their low-cost-production, among others[1]. Over the last 70 years, world annual plastic production has risen from 0.5 million tons to 400 million tons and is expected to double in the next 20 years[2–4]. Today, there are more than 5,000 types of synthetic polymers, 80 % of which are polypropylene (PP), polyethylene (PE), poly(vinyl chloride) (PVC), poly(ethylene terephthalate) (PET) and polystyrene (PS)[5,6]. High demand and production of plastics has increased the amount of plastic waste in the environment, thus causing many problems such as deterioration of the natural balance[2,7]. According to literature[8,9], in 2015, around 9 % of plastic waste had been recycled, 21 % was incinerated, and 79 % was disposed to landfills or improperly disposed.

Plastic particles smaller than 5 mm, known as microplastics (MP), can be classified into primary and secondary MPs. Primary MPs are manufactured plastics added by default to various products at microscopic scale for industrial or domestic use, and secondary MPs are plastics fragmented into much smaller particles due to degradation processes[10]. MPs have become one of the leading environmental threats due to their persistence, ubiquity and intrinsic toxic potential[3]. The potential harm that MPs impose on the environment varies from direct effects (i.e., entanglement and ingestion) to their abil-

*Corresponding author: E-mail: dkucic@fkit.hr

ity to adsorb heavy metals, persistent organic compounds or pharmaceuticals[10]. Studies have shown that MPs exhibit harmful effects on organisms such as reduction of growth and photosynthesis activity, accumulation in the intestine, damage of the intestine and liver, reduction of reproductive ability, physical deteriorations, endocrine disruption, oxidative stress, immune response, disturbing the fine-tuned balance of biological systems and mortality[11–17].

MPs are widely distributed in the environment, i.e., they are present in soils, the marine environment, freshwater systems, as well as in sediments[18]. In the ocean, plastic pieces range at around $0.485–5.4\cdot10^{12}$ in number, weighing $3.54\cdot10^{4}–2.36\cdot10^{5}$ tons[19,20], which could be potentially the largest sink of plastics pollution in the future[21]. The major sources of MPs are varied, with most coming from residential households, landfills, construction, factories, ships, marine platforms, municipal wastewater, activated sludge, and agriculture[22–27].

MP particles are insoluble in water, and have low susceptibility to degradation; therefore, they remain in the environment for long periods, and possess diverse physicochemical properties that determine bioavailability to organisms, most importantly the particle size[28]. Accordingly, investigation into the removal of MPs or plastic waste from the environment is of significant importance. MPs can be biodegraded in the environment (by the action of living organisms), photodegraded (via light radiation), thermo-oxidatively degraded (slow oxidative breakdown at moderate temperature), thermally degraded (high temperature), and hydrolysed (degradation in water with or without contribution of catalysts)[29]. Other strategies of MP removal from water are sorption, filtration methods, coagulation and agglomeration methods, electrocoagulation, photocatalytic degradation, membrane technology, and conventional activated sludge methodology[30]. The use of conventional activated sludge methodology to remove MPs from wastewater can cause an environmental problem. Treatment plants are essentially taking the MPs out of the wastewater and concentrating them in the sludge[31]. Studies of MPs in sewage treatment plants have shown that the retention efficiency depends on the size of the particles, while the shape of the particles is of little importance. More than 99 % of MPs ≥300 μm end up in sludge[32]. Treated activated sludge or biosolids are also commonly used to improve the fertility of agricultural soils. However, the application of biosolids could contaminate the soil through the addition of MPs attached to the sludge particles. However, sludge is not the only problem; sewage treatment plants may play an important role in the release of MPs into the environment, depending on the treatment units used[31,33].

Bioremediation is an efficient, cost effective and eco-friendly removal method that can be combined with other treatment technologies, where microorganisms use their enzymatic apparatus to convert pollutants (in this case, (micro)plastics) to CO_2, H_2O, and inorganic compounds[34,35]. Different enzymes such as laccases, manganese peroxidases, lignin peroxidases, alkane hydroxylases, hydroquinone peroxidase, lipases, cutinases, or carboxylesterases, have been reported to be involved in the biodegradation of PE, PS, PP and PET[36–38]. Biodegradation of polymers is affected by abiotic factors (temperature, pH-value, UV irradiation, salinity, oxygen concentration, moisture content), and biotic factors (number of living cells, presence of single/mixed culture, cell hydrophobicity, diversity of enzymatic system, excretion of extracellular enzymes, possibility of biosurfactants production), as well as the polymer´s chemical and physical properties (surface area, hydrophobicity, morphology, functional groups, molecular weight, flexibility, glass transition temperature, melting temperature, elasticity and crystallinity)[34,39,40]. Abiotic degradation is the critical point on which the rate of MP biodegradation depends. Abiotic degradation produces carbonyl groups that increase hydrophilicity of the polymer and thus increase its availability for biodegradation. Biodegradation of polymers generally includes four phases: biodeterioration, depolymerization, assimilation, and mineralization[34]. To date, only a few research projects have focused on bioremediation of MP-polluted water. Previous research has mainly examined the biodegradability of macroplastics, and the most studied genera of bacteria are *Bacillus* and *Pseudomonas*[18,41,42]. According to literature[18], *Bacillus cereus* degraded 1.6 % of MP-PE, 6.6 % of MP-PET, and 7.4 % of MP-PS in 40 days. More recently, studies have been conducted on the degradation of MP with other bacterial genera and species, such as *Microbulbifer hydrolyticus*[43], *Arthrobacter* sp., *Streptomyces* sp.[44] and *Bacillus megaterium*[45]. Although most research has been conducted with bacteria, fungi can also biodegrade MPs because they have ability to grow on a large spectrum of substrates[46], and are more efficient in biodegradation of complex-structured (e.g., aromatic) polymers[47]. For example, *Fusarium* sp. degraded 9 % of LDPE in 60 days. From the mentioned results, it can be seen that the biodegradation process is slow, and in order to achieve higher efficiency in a shorter time, it is necessary to pre-treat the MPs with physicochemical processes[48]. Furthermore, increasing the efficiency of bioremediation can be achieved by bioaugmentation and biostimulation[49]. Bioaugmentation is the application of indigenous or exogenous microorganisms to hazardous polluted waste sites in order to accelerate the

removal of undesired compounds, in this case MPs. Biostimulation refers to the addition of rate-limiting nutrients like phosphorus, nitrogen, oxygen, and electron donors to severely polluted sites to stimulate the existing bacteria or fungi to biodegrade the hazardous and toxic contaminants[49].

The aim of this study was to examine the possibility of biodegradation of MP-LDPE and MP-PS with particle sizes of 300 – 500 μm, with bacterial cultures of *Bacillus licheniformis*, *Lysinibacillus massiliensis,* and a mixed culture consisting of *Delftia acidovorans* and *Bacillus* sp. during 22 days. The problem encountered in the experiment of MP biodegradation was that it was extremely difficult and complex to monitor the degradation of MP, i.e., it was impossible to determine the mass of MPs at each point of sampling. Therefore, this study took a different approach to monitoring the biodegradation of MPs. The biodegradation of MPs was monitored by determining CFU and the concentration of IC, PO_4^{3-} and K^+. FTIR characterization of MPs was also performed at the end of the experiment.

Materials and methods

Materials

Microplastics

In this work, two types of MPs were used, LDPE (LDPE bags) and PS (disposable cutlery). The structural formula of LDPE and PS are given in Fig. 1(a) and (b). In order to obtain MP particles, the collected plastic materials were firstly cut into smaller pieces with scissors and then ground in a cryo-mill (Retsch, Germany) accompanied with application of liquid nitrogen. The ground plastics were dried in air at room temperature for 48 h, and then sieved on stainless steel screens (W. S. Tyler RX-86-1 sieve shaker, USA) to obtain MP particles in the size range of 300 μm to 500 μm. After sieving, the MP particles were stored in glass bottles. Prior to the experiments, LDPE and PS particles were sterilized in 100-mL flasks containing 70 % ethanol on a rotary shaker (Heidolph unimax 1010, Germany) at 160 rpm for 10 min. Particles were separated from the suspension by vacuum membrane filtration using cellulose nitrate 0.45 μm sterile filters (Ahlstrom ReliaDiscTM), and additionally washed with sterile deionized water. At the end, particles were quantitatively transferred into sterile flasks using sterile working technique.

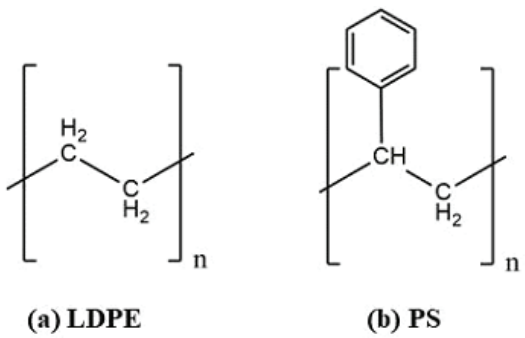

Fig. 1 – *Structural formula of LDPE (a) and PS (b)*

Isolation and identification of MP-degrading bacteria

The bacteria used in this work were isolated from activated sludge and sediment. The activated sludge was collected from the municipal wastewater treatment plant Vrgorac – Split – Dalmatia County, and the sediment from the river Kupa, Karlovac County. Isolation of bacteria from activated sludge and sediment, in the presence of polymers (LDPE and PS) with an area of 1 cm^2, was carried out in 300-mL Erlenmeyer flasks, working volume 100 mL, on a rotary shaker at room temperature and 160 rpm for 54 days. After 54 days, the LDPE and PS samples were placed in 10 mL of sterile physiological NaCl solution (0.9 %), and microbiological analysis was performed by plate count method[50]. Bacterial colonies that were morphologically distinct and dominant on nutrient agar plates (0.5 % peptone, 0.3 % beef extract/yeast extract, 1.5 % agar, 0.5 % sodium chloride, distilled water) were collected and transferred to a new nutrient agar plate and incubated at 37 °C for 24–48 h. Transfer to the new plates was performed repeatedly until a pure isolate was obtained. Light microscope (Olympus B 201, Japan) was used to observe the cell morphology of the isolated bacteria after Gram staining[50], and cell physiological characteristics were determined according to the procedures defined in the Manual of Determinative Bacteriology[51]. In addition, Gram-positive bacteria were also stained with Shaffer-Fulton to determine if they had formed endospores[50]. After Gram and Shaffer-Fulton staining and observation under the microscope, a series of biochemical tests, known as API (Analytical Profile Index, bioMérieux, France), were performed. The final step of identification was matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) based on protein identification of pulsed single ionic analytes (pure microbial culture), coupled with TOF measuring mass analyzer, and exact protein mass was determined.

Characterization of microplastics

ATR-FTIR analysis (Spectrum One, Perkin-Elmer) was carried out to verify the plastic type and visualise morphological changes on LDPE and PS after biodegradation. The FTIR spectra were recorded in the range of 4000–650 cm^{-1}.

Analytical methods

Optical density (OD) of prepared bacterial suspension was determined spectrophotometrically at λ = 600 nm using spectrophotometer Hach Dr/2400, USA[52–54].

Colony-forming units (CFU) of bacteria *Lysinibacillus massiliensis*, *Bacillus* sp., *Bacillus licheniformis* and *Delftia acidovorans* were determined on the general purpose media (nutrient agar) by standard plate count[55]. For plate counting, a dilution series (0.9 mass % of NaCl in aqueous solution) was prepared from each sample. The plates were incubated in 80 % relative humidity at 37 °C for 24–48 h. After incubation, the number of colonies on agar plates was determined. The results were expressed as CFU of bacteria per mL[55].

The concentration of dissolved oxygen (DO) and pH value were measured in Erlenmeyer flasks during biodegradation experiments using DO electrode and pH electrode (WTW Multi 340i, Germany).

Samples collected during the biodegradation process, in which the concentrations of total carbon (TC), total organic carbon (TOC), K^+ and PO_4^{3-} were determined, were filtered through 0.45 μm filter before performing the analysis.

The determination of TOC and TC was conducted on device TOC-V_{CSH}, Shimadzu. An amount of 0.5 mL of the filtered sample was added to the glass vials, and 9.5 mL of Millipore water was added to give a total volume of 10.0 mL for analysis. In the sample in which the TOC was determined, 3 drops of H_2SO_4 were added, while in the sample in which the TC was determined, no acid was added. The concentration of inorganic carbon (IC) was determined from the difference between TC and TOC.

The concentrations of PO_4^{3-} and K^+ were determined using Dionex ICS 3000 dual system with suppressed conductivity. The employed analytical separations of cations were with Dionex IonPac CS16 cation exchange column with its guard column Dionex IonPac CG16. A cation self-regenerating Suppressor (CSRS 300) was used. Anions were separated with Dionex IonPac AS22 with its guard column Dionex IonPac AG22. An anion self-regenerating suppressor (ASRS 300) was used. The injected volume was 25 μL in both systems. The separation of cations was carried out at a flow rate of 1.0 $cm^3\ min^{-1}$ of 20 mmol dm^{-3} MSA, while anions were separated at a flow rate of 1.2 $cm^3\ min^{-1}$ of 4.5 mmol dm^{-3} sodium carbonate/1.4 mmol dm^{-3} sodium bicarbonate eluent. Dionex Chromeleon 6.8 software was employed for instrument control, data collection and processing.

Biodegradation of LDPE and PS in batch conditions

The day before the biodegradation experiment had been set up, bacterial cultures were inoculated onto nutrient agar and incubated at 37 °C for 24 hours. Grown bacterial colonies were harvested with a sterile inoculating loop, pooled, and transferred to a sterile Erlenmeyer flask containing 20 mL of physiological saline (0.9 mass % NaCl) to prepare a thick bacterial suspension.

Biodegradation experiments (P1-1, P1-2 and P1-3) were carried out in sterile 250-mL Erlenmeyer flasks shaken at 160 rpm for 22 days on the thermostatic rotary shaker, Fig. 2 and Table 1. Each reactor was filled with 90 mL of mineral salt medium, 100 mg L^{-1} of MP-LDPE or MP-PS and 10 mL of bacterial suspension. The working volume, *V*r, was 100 mL. The mineral salt medium contained (g L^{-1}): K_2HPO_4 12.5, KH_2PO_4 3.8, $(NH_4)_2SO_4$ 1.0, $MgSO_4 \cdot 7H_2O$ 0.1, H_3BO_3 0.232, $ZnSO_4 \cdot 7H_2O$ 0.174, $FeSO_4(NH_4)_2SO_4 \cdot 6H_20$ 0.116, $Co(NO_3)_2 \cdot 6H_2O$

Table 1 – *Initial conditions for biodegradation process*

Experiment	P1-1		P1-2		P1-3	
Bacterial culture	*Bacillus licheniformis*		*Lysinibacillus massiliensis*		Mixed culture of *Delftia acidovarans* + *Bacillus* sp.	
Microplastics	LDPE	PS	LDPE	PS	LDPE	PS
Particle size of MP/μm	300 – 500					
CFU_0/mL	$6.00 \cdot 10^7$		$1.50 \cdot 10^7$		$1.50 \cdot 10^7$	
$OD_{0\ 600\ nm}$/–	0.33		0.26		0.26	
γ(MP)/mg L^{-1}	100.00					
T_0/°C	25.20					
pH_0/–	7.15					
$\gamma_0(O_2)$/mg L^{-1}	8.33					

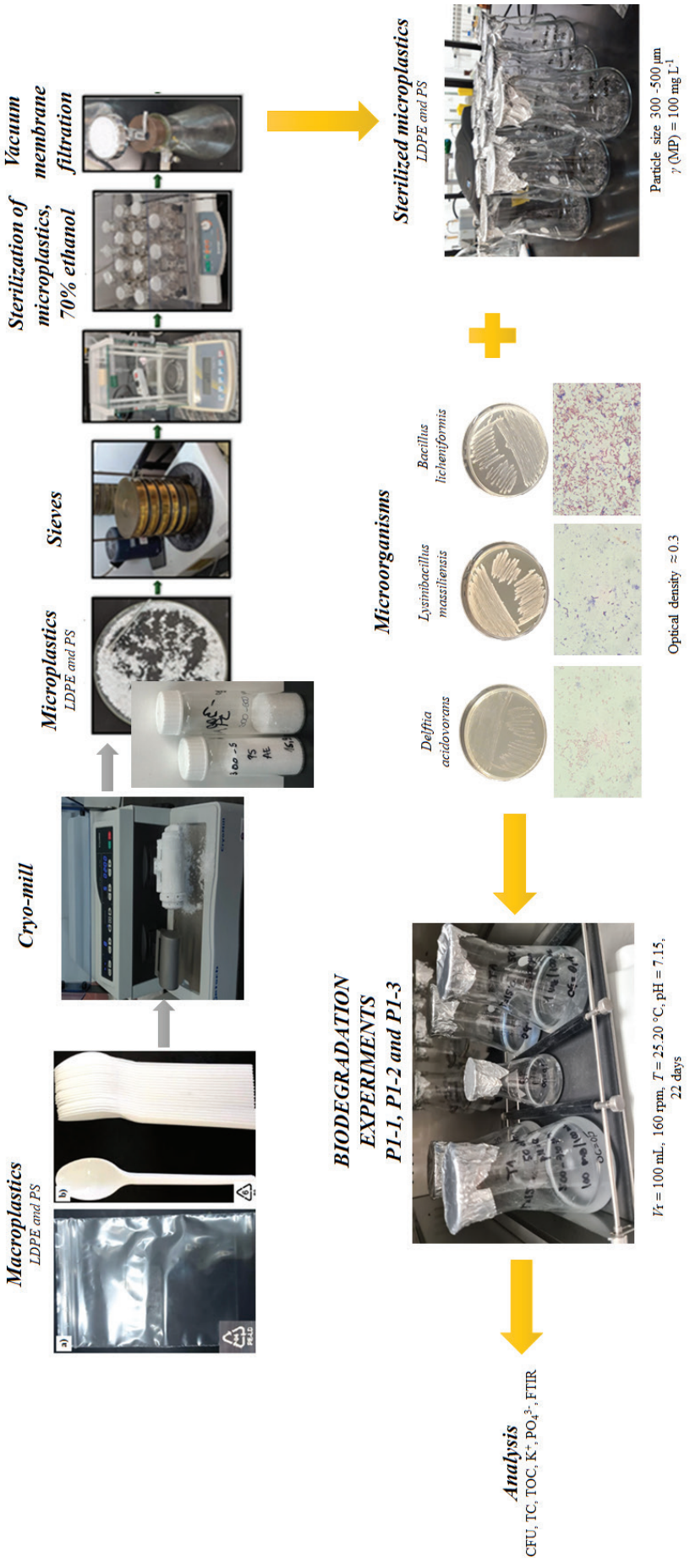

Fig. 2 – *Flow chart of biodegradation process*

0.096, $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ 0.022, $CuSO_4\cdot 5H_2O$ 0.008, $MnSO_4\cdot 4H_2O$ 0.008[56]. Blank experiments were conducted with *Lysinibacillus massiliensis*, *Bacillus licheniformis,* and a mixed culture of *Bacillus* sp. and *Delftia acidovorans* to assess biodegradability. Blank experiments contained no MPs. All experiments were performed in duplicate. Samples were taken at regular time intervals and CFU, concentrations of DO, TC, TOC, IC, PO_4^{3-}, K^+ were determined for each experiment (P1-1, P1-2 and P1-3). At the end of the experiment, the FTIR analysis of MP-LDPE and MP-PS was performed.

Results and discussion

In recent years, more and more attention has been paid to MPs and their potential harmful effects on humans and the environment. MPs are most often released into the environment by the decomposition of improperly disposed plastic waste or personal care products. Due to the growing awareness of potentially harmful effects, many organizations are promoting proper waste disposal. However, plastics derived from mineral oil have been accumulating in the environment for decades. As previously mentioned, various methods are used to remove MPs, including sorption on green algae (efficiency 94.5 %)[57], membrane bioreactors (efficiency >99 %)[58], conventional activated sludge (efficiency 98 %)[58], wastewater treatment plant (efficiency >96 %)[59], coagulation and agglomeration processes (efficiency 61 %)[60], photocatalytic degradation[61] and biodegradation[41,42,56] (depending on the type of microbial community, efficiency >20). Each of these processes has advantages and disadvantages, and by combining them, an environmentally friendly and economically sustainable process can be achieved. However, to achieve this, it is necessary to study each process individually. The advantage of using the biodegradation process to remove MPs is simplicity and safety for widespread use, low operating costs, practical applicability in different environments, flexibility in handling a wide range of wastewater characteristics and flows[30]. There are still various questions about the rate of MPs biodegradation in terrestrial and especially in aquatic systems.

After 54 days, the dominant bacteria on the nutrient agar plates were isolated and identified. Based on biochemical tests, known as API and MALDI-TOF MS analysis, the identified bacteria were *Lysinibacillus massiliensis*, *Bacillu*s sp., *Bacillus licheniformis* and *Delftia acidovorans*. Gram-positive bacteria, *Lysinibacillus massiliensis* and *Bacillus* sp., were isolated from activated sludge, and Gram-positive bacteria *Bacillus licheniformis* and Gram-negative bacteria *Delftia acidovorans* were isolated from sediment. Shaffer-Fulton staining showed that *Lysinibacillus massiliensis*, *Bacillu*s sp., and *Bacillus licheniformis* had formed endospores, which was consistent with the literature[62].

Changes in CFU during biodegradation of MP-LDPE and MP-PS with *Bacillus licheniformis*, *Lysinibacillus massiliensis,* and mixed bacterial culture of *Bacillus* sp. and *Delftia acidovorans* are shown in Fig. 3(a-c). In all three cases, it was observed that the number of colonies formed by the bacteria, *Bacillus licheniformis*, *Lysinibacillus massiliensis* and mixed bacterial culture of *Bacillus* sp. and *Delftia acidovorans*, was higher compared to the blank, indicating that the tested bacteria used LDPE and PS as carbon and energy source[63,64]. In the first seven days, there was an exponential growth of bacteria in both MP-LDPE and MP-PS (Fig. 3(a-c)), after which a stationary phase occurred, which lasted until the end of the process. According to the literature, the stationary phase may last longer than 90 days[18]. The concentration of dissolved oxygen also decreased with increasing CFU in all three cases (Fig. 4(a-c)), while it slightly increased in the blank experiment. CFU in the blank (in all three cases) increased slowly during the first seven days, and then decreased slightly until the end of the process. The tested bacterial cultures, *Bacillus licheniformis*, *Lysinibacillus massiliensis* and *Bacillus* sp., form endospores that enable them to survive under extreme conditions, such as high temperature, low pH, and under conditions of starvation[55]. Dead cells can also serve as a carbon source for living cells. It can be seen from Figs. 3 and 4, that the greatest increase in CFU as well as the decrease in concentration of dissolved oxygen was in MP-LDPE for all three cases. MP-LDPE and MP-PS are the most common plastic with a full C-C backbone, but PS has phenyl rings and is considered to be the thermoplastic polymer most resistant to biodegradation[65]. According to literature[66] the "rapid" biodegradation observed with MP-LDPE does not occur with PS. According to research[67], bacteria possessing enzyme oxidase – laccases, such as *B. licheniformis*, release this enzyme leading to cleavage of PE chains to low molecular weight compounds. The resulting compounds are transported into the cell where they undergo mineralization, i.e., biodegradation takes place[67]. Research[68] has shown that *Bacillus cereus*, which contains enzymes such as nitrate reductase and catalase, can partially degrade MP-LDPE over 90 days. In addition, studies by Mukherjee *et al.*[69] showed that the bacteria *Bacillus licheniformis* and *Lysinibacillus fusiformis* can biodegrade MP-LDPE. The highest decrease in dissolved oxygen concentration as well as the increase in CFU during biodegradation

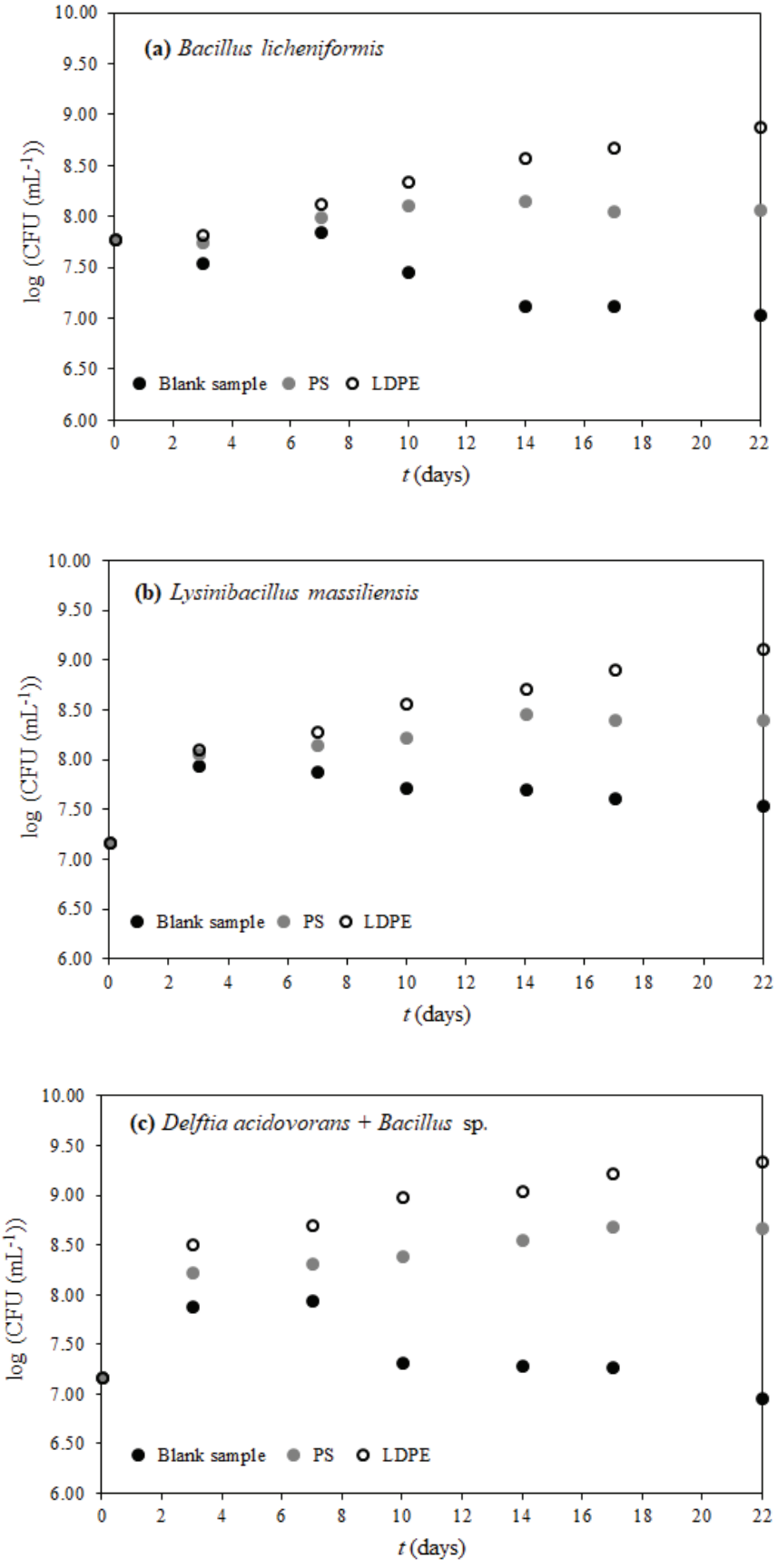

Fig. 3 – *Changes in CFU during biodegradation of MP-LDPE and MP-PS with bacterial culture Bacillus licheniformis (a), Lysinibacillus massiliensis (b), and mixed bacterial culture of Bacillus sp. and Delftia acidovorans (c) in sterile Erlenmeyer flasks during 22 days (Vr = 100 mL)*

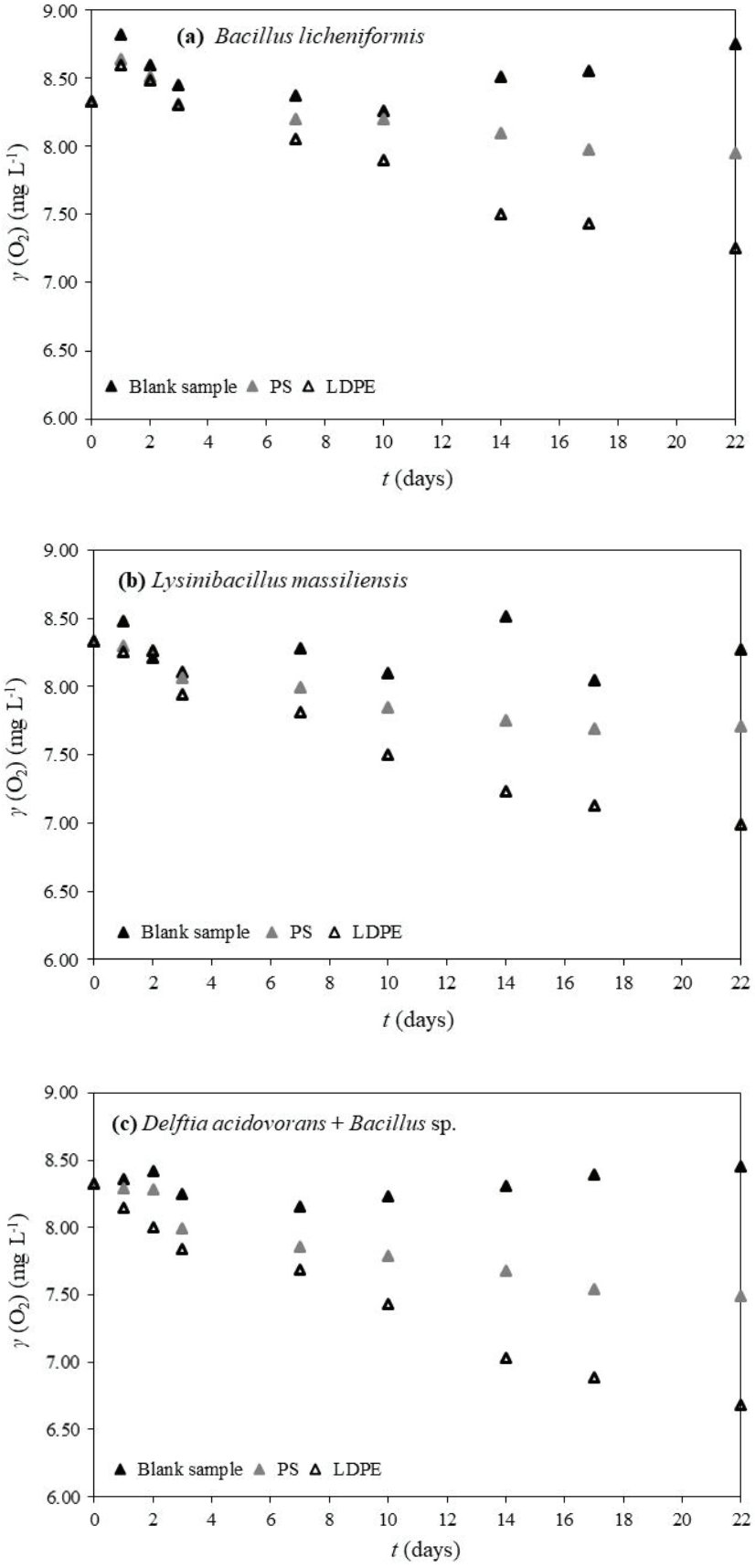

Fig. 4 – *Changes in concentration of dissolved oxygen during biodegradation of MP-LDPE and MP-PS with bacterial culture Bacillus licheniformis (a), Lysinibacillus massiliensis (b), and mixed bacterial culture of Bacillus sp. and Delftia acidovorans (c) in sterile Erlenmeyer flasks during 22 days (Vr = 100 mL)*

of MP-LDPE and MP-PS was observed in the sample with mixed bacterial culture, *Bacillus* sp. and *Delftia acidovorans* (Figs. 3 and 4), which is in line with published studies[70,71]. Mixed bacterial cultures have a wider range of enzymes and are capable of degrading a greater number of compounds than pure cultures. However, in a mixed culture, there may be competition for the substrate, so that one culture predominates over another. In this experiment, the proportion of one species relative to the other had not changed during the 22 days of the process. The biodegradation of MP-LDPE with *Delftia* was previously studied by Peixoto *et al.*[72] The obtained results showed that no significant cell death of *Delftia* occurred during 20 days of exposure to LDPE. LDPE was the only source of carbon; therefore, the cells adsorbed on the surface secreted enzymes and performed the necessary reactions to assimilate carbon from LDPE. In this way, it was proven that *Delftia* can use LDPE as a carbon source. After 90 days of the experiment, a large number of living cells were also present[72].

With the increase in CFU and decrease in dissolved oxygen concentration, the concentrations TC, TOC and IC increased in all three samples (Figs. 5–7), confirming the biodegradation of MP-LDPE and MP-PS. An increase in the TOC concentrations (Figs. 5b – 7b) in MP-LDPE and MP-PS samples may indicate the release of additives from the MP surface as well as organic products resulting from disturbances in the structure of MP-PS and MP-LDPE, which was not the case in the blank sample. The concentration of TOC in blank sample had not changed during 22 days, Figs. 5–7. The concentration of IC (Figs. 5c – 7c) changed slightly in the blank sample, i.e., a slight increase was observed when CFU began to decrease (Fig. 3a–c), indicating lysis of bacterial cells[55]. As dying cells lyse or break, they release their contents into the environment, making these nutrients available to other bacteria[55]. This was also confirmed by an increase in the concentration of phosphate and potassium in the blank sample (Figs. 8 and 9). Indeed, a bacterial cell, like all other cells, is composed of carbon, hydrogen, oxygen, nitrogen, phosphorus, and other elements[55]. The concentration of IC in the samples MP-LDPE and MP-PS increased from the third to the tenth day (Figs. 5c – 7c), as did CFU (Fig. 3a–c) and TOC (Figs. 5b – 7b), indicating that degradation of MP-LDPE and MP-PS had occurred, i.e., CO_2 was produced as one of the products of biodegradation[73]. After day 10, the concentration in all samples fluctuated slightly, as did the CFU and dissolved oxygen concentration. While IC concentration increased, phosphate and potassium concentrations decreased (Figs. 8 and 9). According to the obtained results, it can be assumed that the cells consumed inorganic phosphate and potassium from the mineral medium. Moreover, the increase in IC concentration, as well as the decrease in phosphate concentration was more evident in samples with MP-LDPE and mixed bacterial culture *Bacillus* sp. *and Delftia acidovorans*. Phosphorus is an essential nutrient for microbes as it is one of the macronutrients present in all cells as part of the macroenergetic compounds adenosine diphosphate (ADP) and adenosine triphosphate (ATP)[74]. Bacteria require phosphorus for the biosynthesis of nucleic acids, lipopolysaccharides and phospholipids[74,75]. The major fraction of phosphorus in bacterial cells is DNA + RNA + lipids, which account for about 60 % of the total cell phosphorus, other fractions are cytoplasmic phosphate (organic and inorganic) and polyphosphate[76]. According to Anderson and Domsch[77], the ratio of C:P in bacterial cells is 17.

Potassium is the major monovalent intracellular cation in cells, and its uptake is essential for all living organisms. It has many key functions within bacterial cells: potassium is required for the activity of intracellular enzymes, acts as an intracellular second messenger, and is involved in maintaining constant internal pH and membrane potential. In addition, potassium plays an important function as an osmotic solute[78].

Table 2 lists the bacteria that have the potential to biodegrade MP-LDPE and MP-PS. Previous studies have shown that MP-LDPE is more biodegradable than MP-PS, as in this study. The longer the biodegradation process of MP-LDPE and MP-PS, the more efficient the biodegradation. Comparison of the genera *Bacillus* and *Pseudomonas* revealed that the genus *Pseudomonas* degraded both polymer materials more efficiently in a shorter exposure time. In addition, the mixed bacterial culture degraded MP-LDPE better in a shorter time than the pure bacterial culture, which is consistent with the results of this study. Mixed bacterial culture can have significantly greater effects on film hydrophilicity, surface chemistry, and mineralization of MPs than pure bacterial cultures[44,79,80].

Fig. 10(a) shows the FTIR – ATR spectrum of MP-LDPE before biodegradation. Characteristic peaks for LDPE occur at wavelengths of 2900 cm^{-1}, 2800 cm^{-1}, 1500 cm^{-1}, 1450 cm^{-1}, and 750 cm^{-1} which is in agreement with the literature[81,82]. Fig. 10(b) – (d) shows the obtained FTIR – ATR spectra after the MP-LDPE biodegradation process with *Bacillus licheniformis*, *Lysinibacillus massiliensis* and mixed bacterial culture of *Bacillus* sp. and *Delftia acidovorans*. The spectra obtained differ from the original MP-LDPE spectrum. Although all peaks characteristic of MP-LDPE were still present, there was a visible decrease in the intensity of all peaks. In addition, new groups developed. A peak was ob-

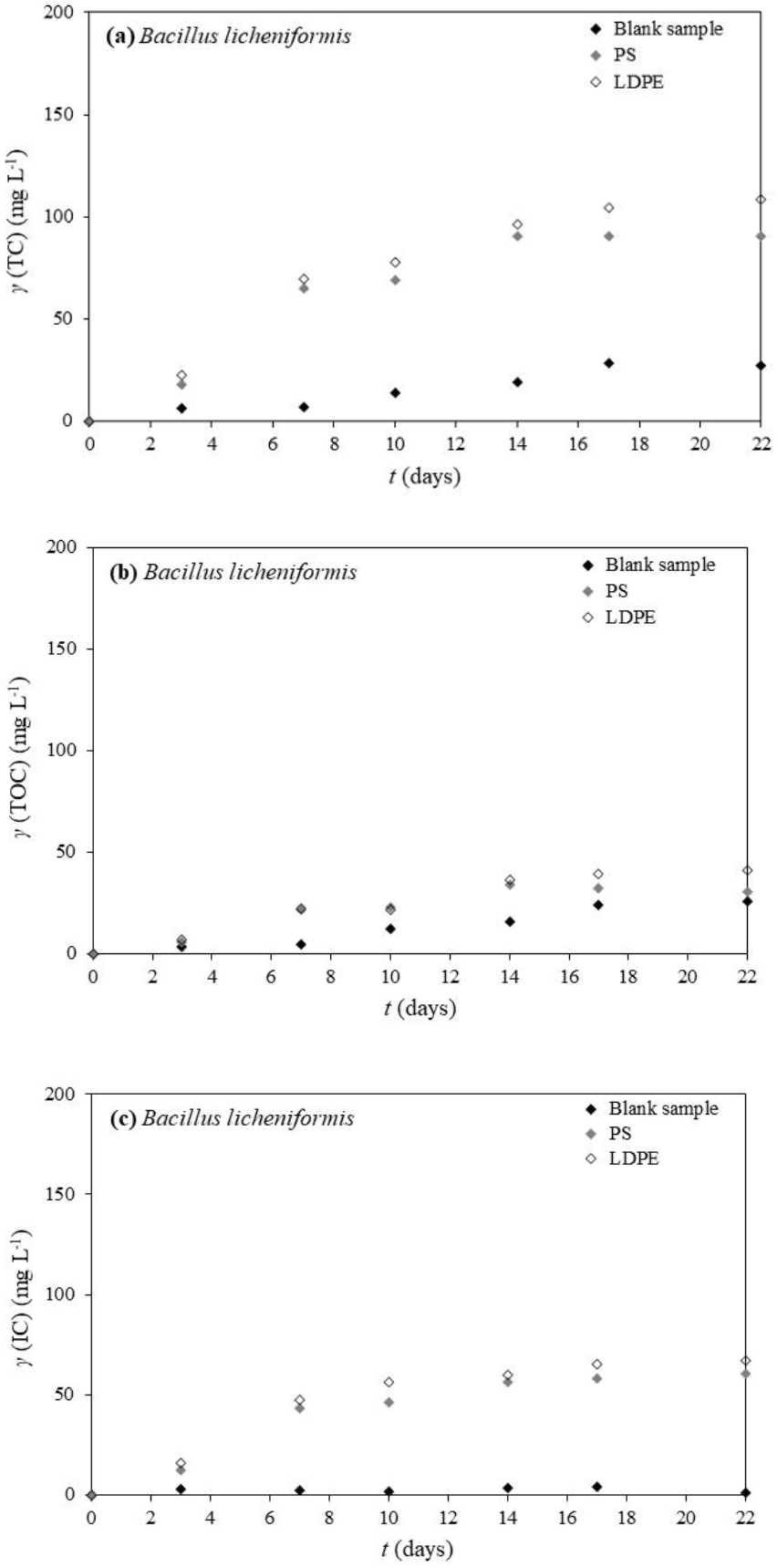

Fig. 5 – *Changes in TC (a), TOC (b) and IC (c) concentration during biodegradation of MP-LDPE and MP-PS with bacterial culture Bacillus licheniformis in sterile Erlenmeyer flasks during 22 days (Vr = 100 mL)*

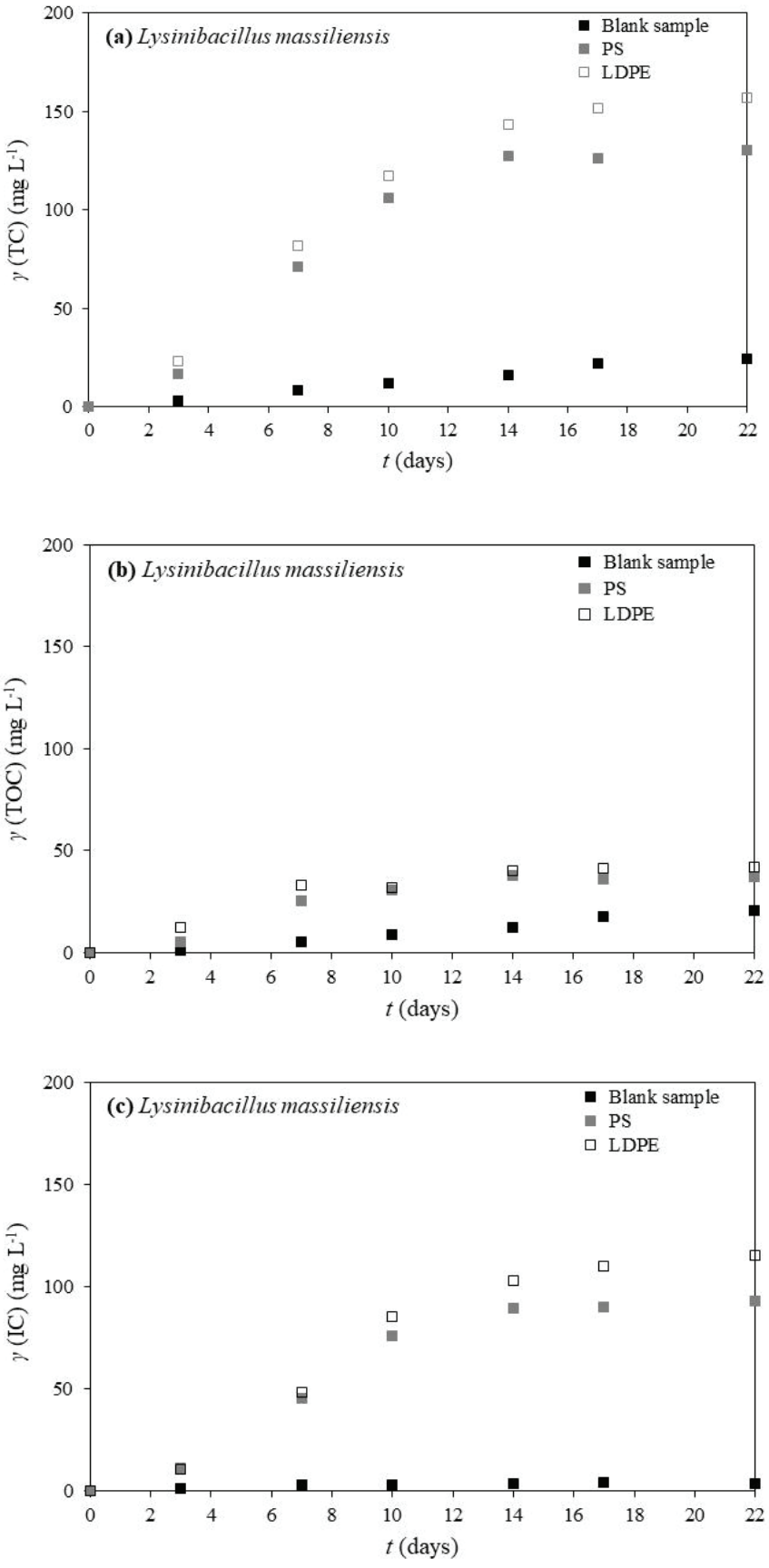

Fig. 6 – *Changes in TC (a), TOC (b) and IC (c) concentration during biodegradation of MP-LDPE and MP-PS with bacterial culture Lysinibacillus massiliensis in sterile Erlenmeyer flasks during 22 days (Vr = 100 mL)*

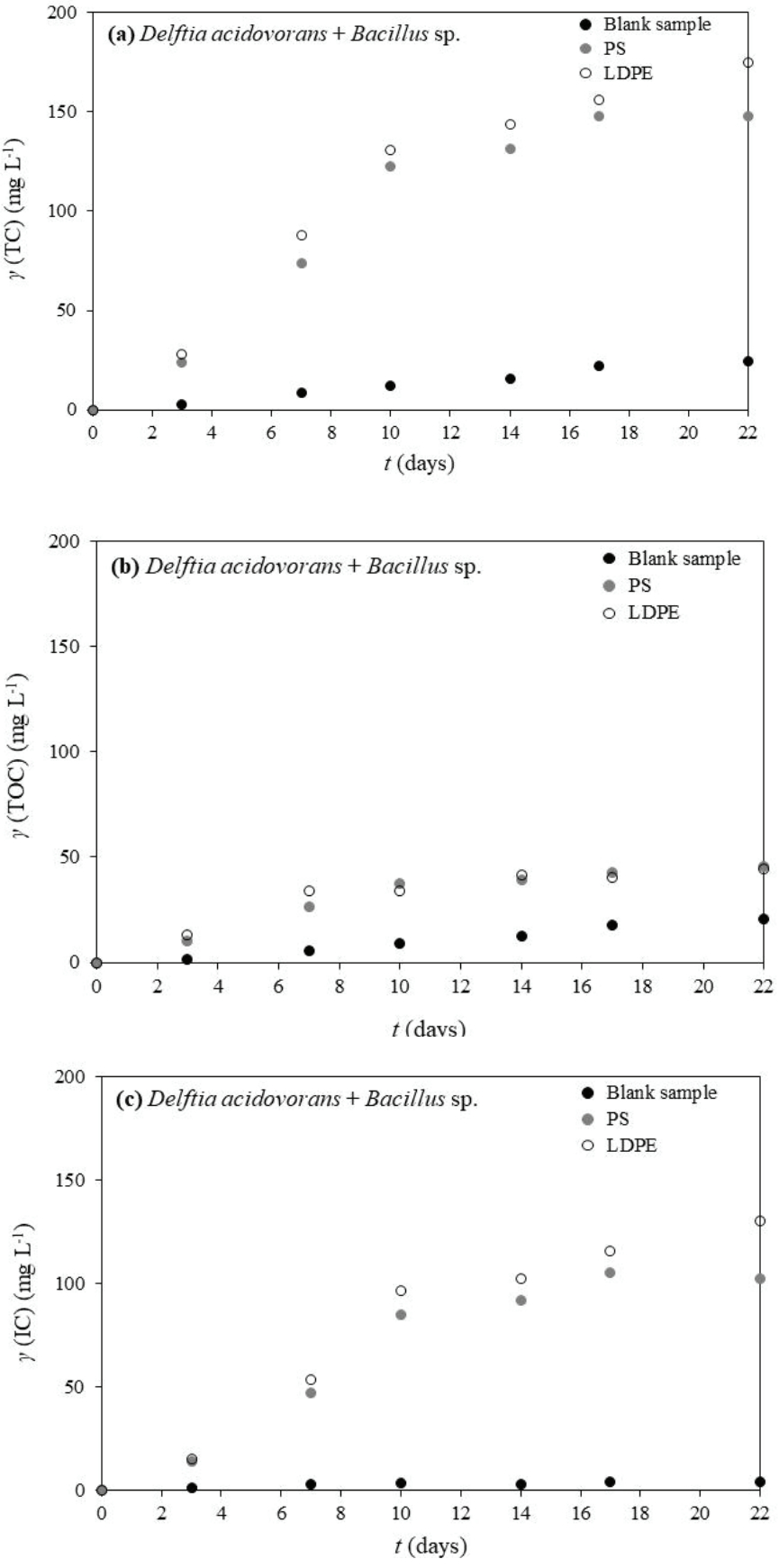

Fig. 7 – *Changes in TC (a), TOC (b) and IC (c) concentration during biodegradation of MP-LDPE and MP-PS with mixed bacterial culture of Bacillus sp. and Delftia acidovorans in sterile Erlenmeyer flasks during 22 days (Vr = 100 mL)*

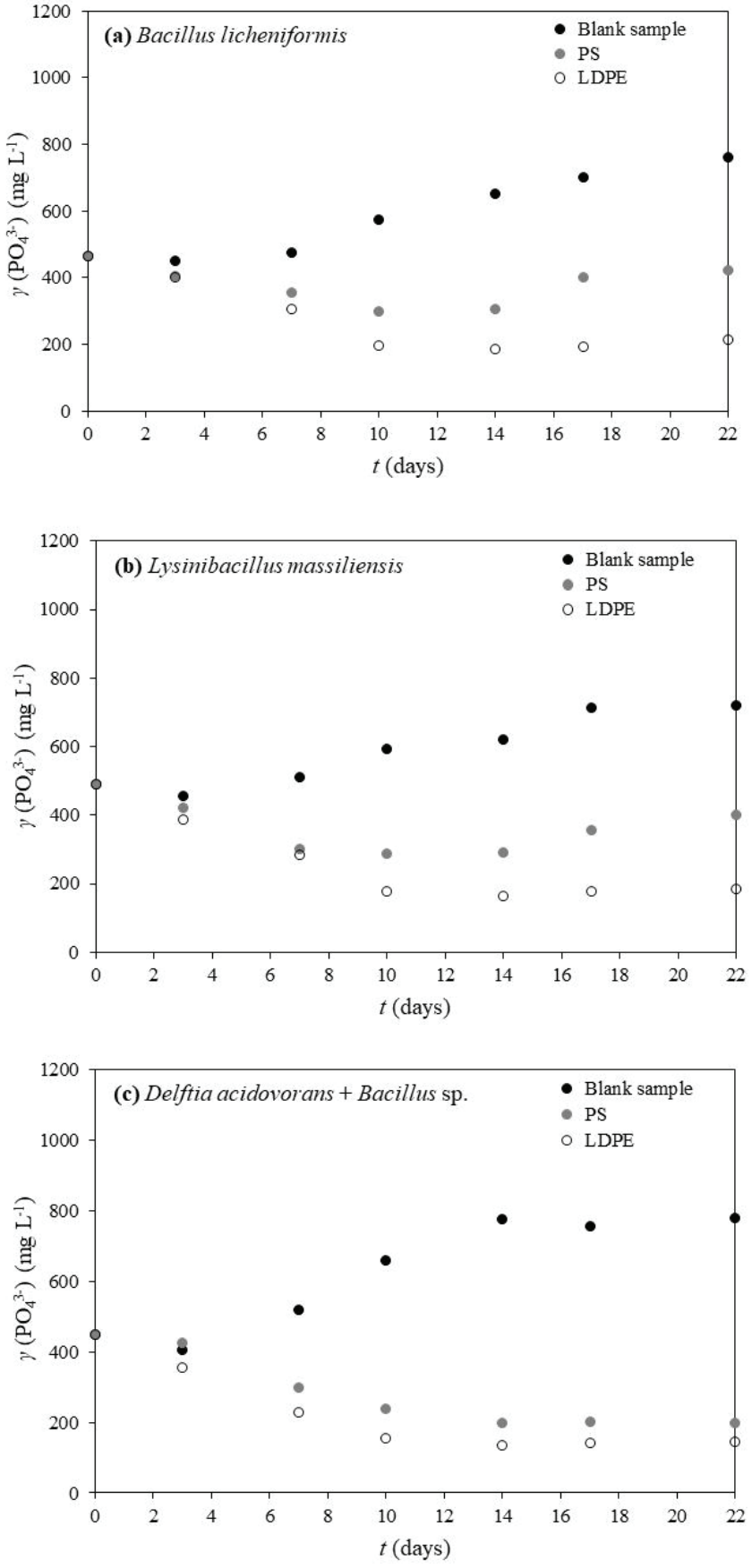

Fig. 8 – *Changes in phosphate concentration during biodegradation of MP-LDPE and MP-PS with bacterial culture Bacillus licheniformis (a), Lysinibacillus massiliensis (b), and mixed bacterial culture of Bacillus sp. and Delftia acidovorans (c) in sterile Erlenmeyer flasks during 22 days (Vr = 100 mL)*

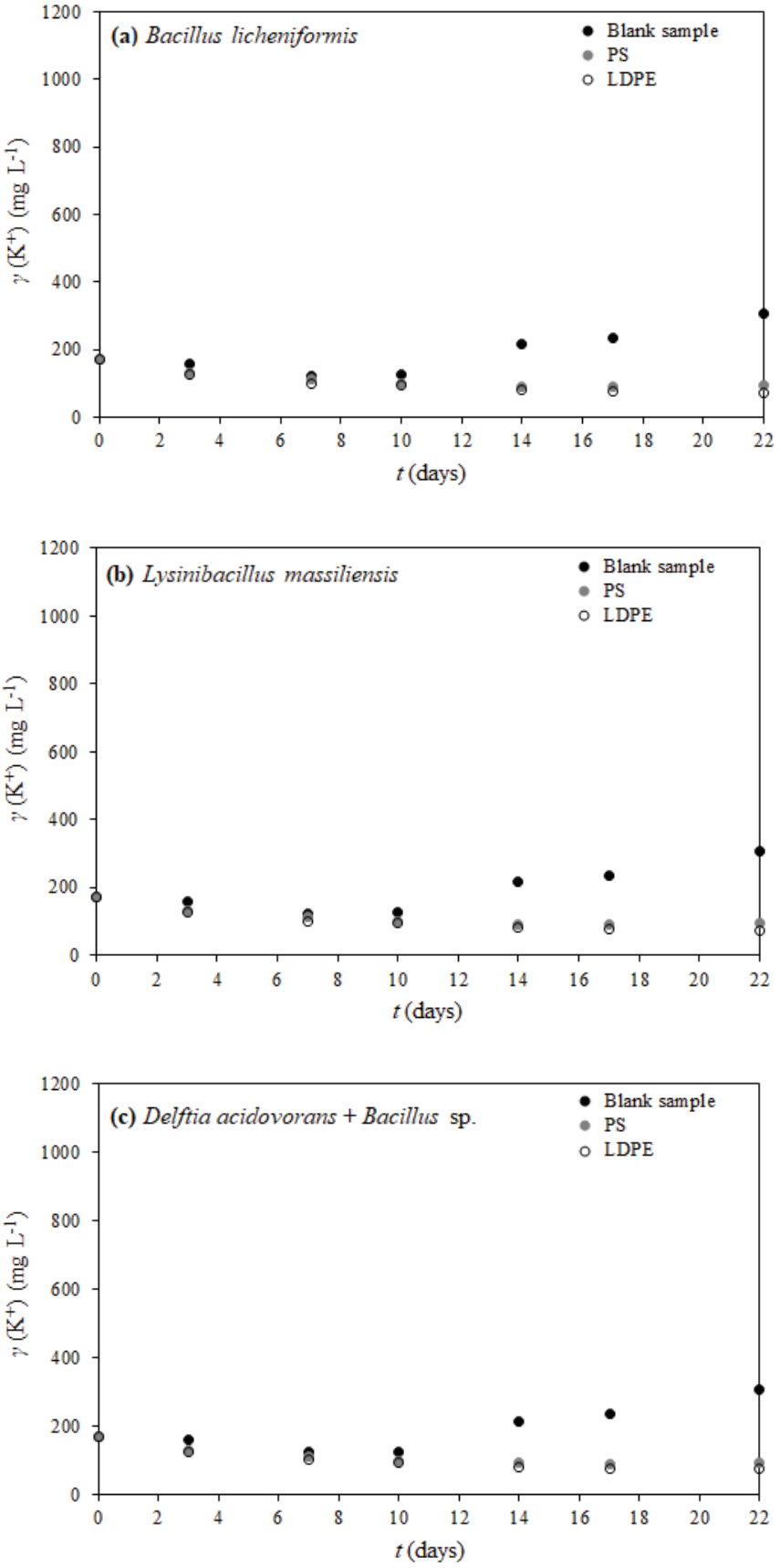

Fig. 9 – *Changes in potassium concentration during biodegradation of MP-LDPE and MP-PS with bacterial culture Bacillus licheniformis (a), Lysinibacillus massiliensis (b), and mixed bacterial culture of Bacillus sp. and Delftia acidovorans (c) in sterile Erlenmeyer flasks during 22 days (Vr = 100 mL)*

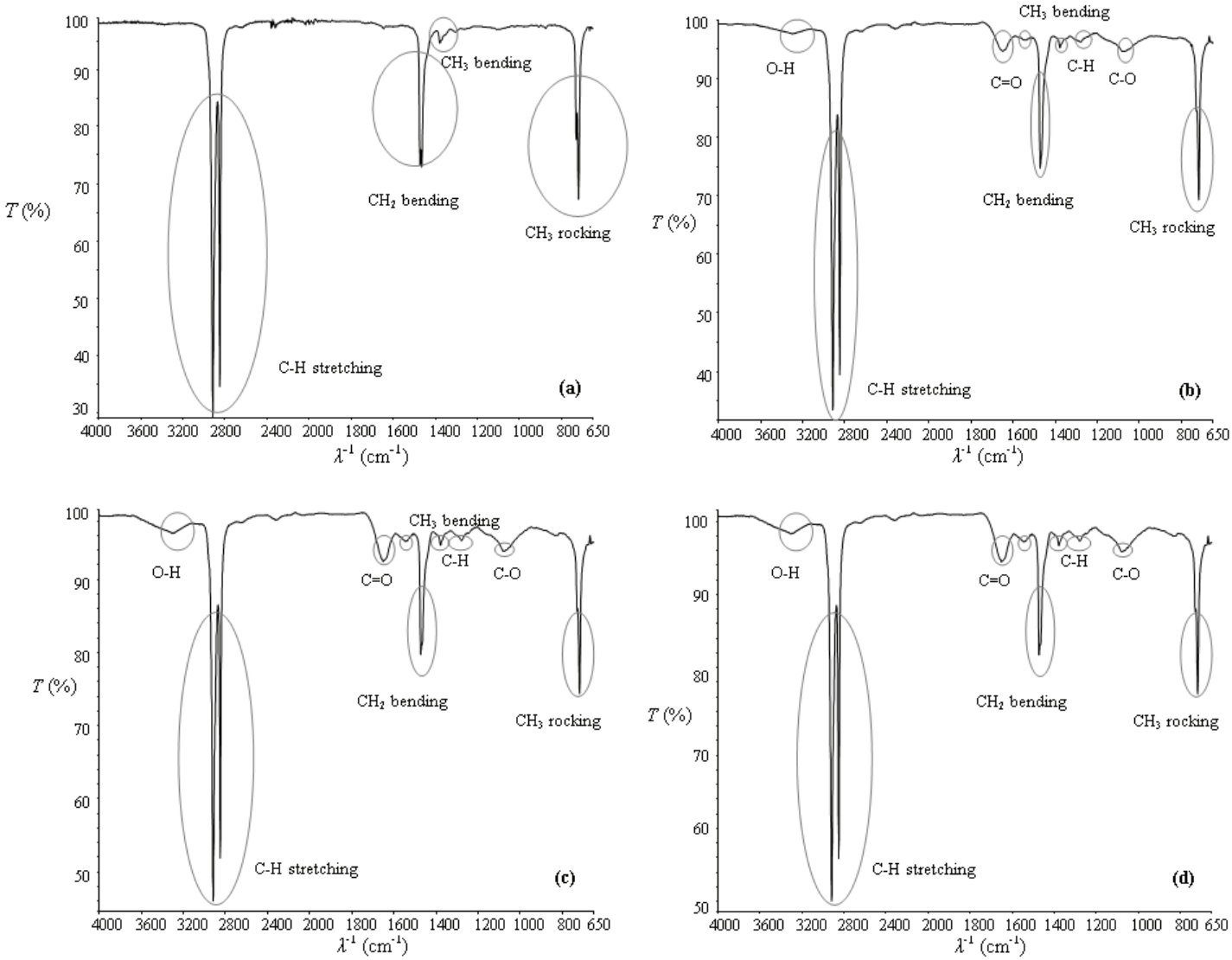

Fig. 10 – *FTIR-ATR spectrum before (a), and after biodegradation of MP-LDPE with bacterial culture Bacillus licheniformis (b), Lysinibacillus massiliensis (c), and mixed bacterial culture of Bacillus sp. and Delftia acidovorans (d)*

served at ~ 3300 cm^{-1} characterizing the stretching of the O–H group in the alcohol. This peak was present in all samples after biodegradation, therefore it can be assumed that it was not formed during the biodegradation process, but during washing of MP-LDPE with ethanol (w = 70 %) before analysis. The next newly-formed peak is located at ~ 1680 cm^{-1} and is characterized by the stretching of C=O bonds in aldehydes or ketones. Indeed, the formation of carbonyl groups, mostly belonging to ketones, is the most common indicator that MP biodegradation has occurred[82,83]. The formation of new groups, as shown by the peak at a wavelength of 1350 cm^{-1} (C–H stretching), also confirms the biodegradation of MP-LDPE, i.e., cleavage of the MP-LDPE polymer chain into smaller parts. A peak was formed at a wavelength of 1150 cm^{-1} characterizing the stretching of C–O bonds in ethers, esters, or carboxylic acids. FTIR analysis showed that the increases in the intensity of the new bands in the ranges of 3700–3000 cm^{-1}, 1700–1500 cm^{-1}, and 1200–950 cm^{-1} can be attributed to the hydroperoxide and hydroxyl groups, carbonyl groups, and double bonds, respectively, indicating the oxidative degradation of polyethylene microplastics. Considering the obtained results, it can be assumed that the mixed bacterial culture, *Bacillus* sp. *and Delftia acidovorans*, degraded MP-LDPE better than pure bacterial cultures due to a greater reduction in peak intensity and spectral deformation.

Fig. 11(a) shows the FTIR – ATR spectrum of MP-PS before biodegradation. The MP-PS spectrum is characterized by a peak at wavelength 3000 cm^{-1} belonging to the C–H stretching of the aromatic group. The peak at ~ 2850 cm^{-1} represents the C–H elongation, and the peaks around ~ 1500–1550 cm^{-1} are characteristic of the elongation of the aromatic ring. The peaks at ~ 1450 cm^{-1} and ~ 1050 cm^{-1}

Table 2 – *List of the bacteria with potential to biodegrade LDPE and PS*

Polymer	Microorganisms	Weight loss/%	Exposure period	References
PE	*Bacillus cereus*	35.7	16 weeks	41
		1.6	40 days	18
	Bacillus gottheilii	6.2	40 days	18
	Brevibacillus borstelensis	20.3	16 weeks	41
	Bacillus sp. and *Paenibacillus* sp.	14.7	60 days	74
	Pseudomonas aeruginosa and *Brevibacterium* sp.	7.31	30 days	75
	Pseudomonas aeruginosa	50.5	60 days	77
		20.0	120 days	56
	Pseudomonas putida	9.0	120 days	56
	Pseudomonas syringae	11.3		
PS	*Bacillus cereus*	7.4	40 days	18
	Bacillus gottheilii	5.8		
	Bacillus spp.	23.7	30 days	42
	Pseudomonas spp.	<10	30 days	42
	Rhodococcus ruber	0.8	8 weeks	60

represent the CH_2 bending, i.e., the bending of the C–H bonds in the aromatic ring. The bending of the aromatic groups is evident from the peaks at ~ 670, ~ 780 and ~ 850 cm^{-1}. The spectrum was analysed according to the literature data[81,84]. Fig. 11(b) – (d) shows the obtained FTIR – ATR spectra after the MP-PS biodegradation process with *Bacillus licheniformis*, *Lysinibacillus massiliensis* and a mixed bacterial culture of *Bacillus* sp. and *Delftia acidovorans*. The spectra obtained differ from the original MP-PS spectrum. Although all peaks characteristic of MP-PS were still present, the intensity of all peaks had visibly decreased. The sharpest decrease in intensity is visible in the peaks at wavelengths 700 and 1050 cm^{-1}, while the peak at 2850 cm^{-1} shifted to a smaller wavelength (2750 cm^{-1}). The decrease in intensity indicated that the bacterial culture had degraded the polymer into smaller intermediates and used it as a carbon source. In addition, many new peaks had appeared. Peaks of low intensity were observed at wavelengths 3500 cm^{-1} and 3300 cm^{-1} which are characteristic of the stretching of the O–H group in alcohol that was also observed in MP-LDPE. Newly-formed peaks were observed at wavelengths of 2300 cm^{-1}, 1600 cm^{-1} and 1050 cm^{-1} and are characterized by elongation O=C=O, C=C and CO–O–CO in CO_2, alkene and aldehydes, respectively[82,83,85]. As for MP-LDPE, a new peak had also formed at wavelength 1350 cm^{-1} (C–H stretching) in MP-PS, confirming the biodegradation of PS, i.e., the cleavage of the PS polymer chain into smaller parts. Based on the obtained results, it can be considered that the mixed bacterial culture degraded PS the best. Visible are the greatest deformations of the spectrum and a decrease in the intensity of the peaks.

However, as previously mentioned, during the biodegradation of MP-LDPE, characteristic groups had also emerged, indicating the biodegradation of MPs, along with a decrease in peak intensity; therefore, it uncertain whether the mixed culture better degraded MP-PS or MP-LDPE.

Conclusion

Synthetic polymers are one of the most significant pollutants in the aquatic environment. Most studies are focused on MPs. The presence of MPs in the environment can have negative effects on the entire ecosystem. Due to high molecular weight, chemical inertness, and lack of functional groups, biodegradation of long polyolefin chains is limited. However, bioaugmentation of microorganisms can increase the biodegradability of MPs.

In this work, the biodegradation of MP-PS and MP-LDPE was studied using pure bacterial cultures, *Bacillus licheniformis* and *Lysinibacillus massiliensis*, and a mixed bacterial culture of *Bacillus* sp. and *Delftia acidovarans* for 22 days. CFU, IC,

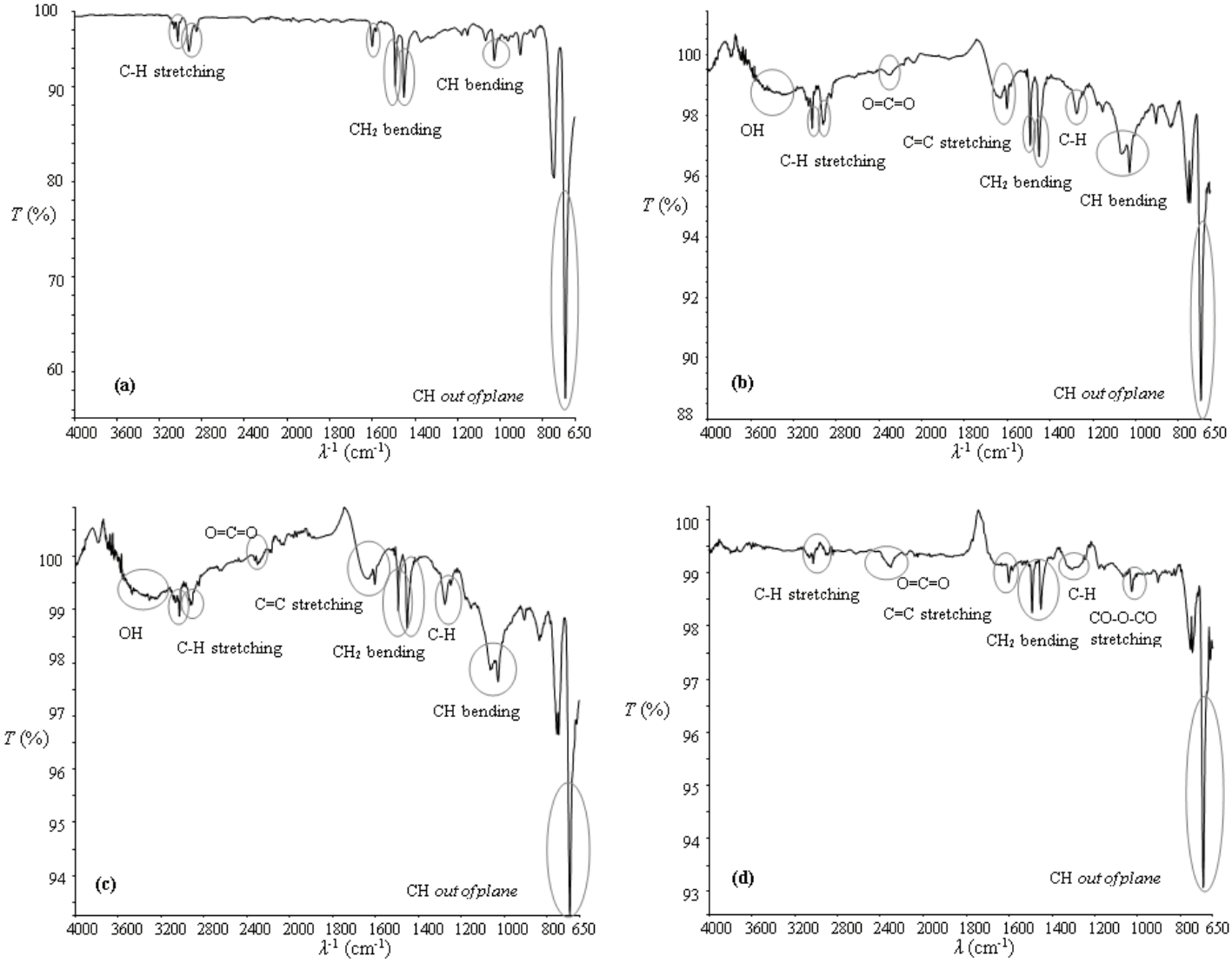

Fig. 11 – *FTIR-ATR spectrum before (a) and after biodegradation of MP-PS with bacterial culture Bacillus licheniformis (b), Lysinibacillus massiliensis (c) and mixed bacterial culture of Bacillus sp. and Delftia acidovorans (d)*

TOC and FTIR show that mixed bacterial cultures degraded MP-LDPE and MP-PS better than pure bacterial cultures, and the biodegradation efficiency was higher for MP-LDPE. The increase in TOC concentration indicated that there was a release of additives from the surface of MP-LDPE and MP-PS and disruption of its structure. With the increase in CFU, the concentration of IC also increased, indicating that biodegradation of MPs was indeed occurring.

The appearance of hydroperoxide and hydroxyl groups, carbonyl groups and characteristic double-bond bands in the FTIR patterns of the treated MPs indicated the oxidative mechanism for biodegradation. Plastics are usually not easily biodegradable, so they may persist for a long time. At advanced stages of abiotic degradation, the plastic develops surface features, becomes weak, and starts to lose its mechanical integrity. Thus, to achieve the highest possible degradation efficiency for MPs, they should be subjected to abiotic degradation before biodegradation.

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