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Pseudomonas alloputida and *Pseudomonas taiwanensis* in a process of degradation of polymer materials

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ABSTRACT:
The literature indicates that the source of microorganisms with the potential of polymer biodegradation may be in particular anthropogenically degraded areas such as landfills, contaminated areas of former factories and processing plants. These environments often contain polymer waste, especially in the form of microplastics. Biodegradation is a significant microbiological process in which bacteria or fungi with hydrolytic activity are essential. Polylactide (PLA) is one of the most commonly used biodegradable polymer materials. Its biodegradation in mesophilic conditions is difficult due to too low temperature. In order to improve and accelerate this process, microorganisms that can be effective in mesophilic biodegradation of PLA were selected. Isolated environmental bacteria, *Pseudomonas alloputida* and *Pseudomonas taiwanensis*, were identified as high hydrolytic towards complex organic compounds and polymer materials. They accelerate the degradation of PLA, as demonstrated by the results of infrared Fourier analysis (FTIR-ATR), SEM-EDX microscopic analysis and physico-mechanical values. Obtained results indicate the application potential of the tested bacteria to significantly decrease soil and compost contamination..

Keywords: biodegradation, polylactide, hydrolytic activity, environmental bacteria

1. Introduction

The sustainability and circularity in the European Union can be achieved with the help of the increasing use of renewable and bio-based polymer plastics. This is due to the reduction in the use of fossil fuels and the environmental pollution caused by the generation of microplastics. The most advantageous combination is plastics that are both bio-based and biodegradable, and additionally compostable (European Comission 2023). PLA is one of the most used biodegradable and compostable polymer materials, especially in packaging. The biodegradation process of PLA involves the initiation of hydrolysis, during which oligo- and monomers are released as a result of chain cleavage. Smaller PLA molecules are more susceptible to enzymes produced by microorganisms found in the environment (Zaaba 2020). Its biodegradation in mesophilic conditions is difficult due to too low temperature (Janczak 2018). Therefore, many researchers consider this material to be only compostable under conditions of high temperature and continuous aeration.

The literature indicates that the source of microorganisms with the hydrolytic activity towards complex and toxic substances may be in anthropogenically degraded areas. These environments often contain polymer waste, so it is highly probable that there are microorganisms assimilating carbon from them (Nourollahi 2019, Jalani 2020). Such

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isolated mesophilic microorganisms can utilize a whole range of complex compounds, such as heavy metal compounds, aliphatic and aromatic hydrocarbons, including benzene, paraffin, naphthalene, diesel oil, crude oil and petroleum (Margesin, 2003, Spini 2018). Thus, the bioaugmentation of microorganisms that can carry out the biodegradation process of PLA and other polymeric plastics in soil and compost environments is a valuable treatment to improve the bioremediation processes of contaminated areas.

Among the environmental bacteria that have a beneficial effect on the environment, we can distinguish a variety of strains generally classified as *Pseudomonas putida*. These are microorganisms of significant scientific importance, both in biotechnology and in plant cultivation. It is known that among *P. putida* are bacteria that promote plant growth and bacteria that protect plants from its pathogens. According to the literature, the animal pathogens found among these bacteria have not yet been identified. Among this group, *P. alloputida* has a documented ability to bioremediate toluene, which is toxic to human health. (Passarelli-Araujo 2021, Timmis 2002).

Another example of a bacteria capable of producing a wide variety of hydrolases is *Pseudomonas taiwanensis*, which hydrolyzes, for instance *DL*-lactic acid (Wang L. 2010), chitin (Wang S. 2010.) and cellulose (Zulaika 2022). Researchers demonstrated its strong antifungal activity against plant pathogens (Afzal 2017). As one of the few known bacterial strains, *P. taiwanensis* is also able to dispose of xylose when it is the only source of carbon (Wordofa 2018).

Both *P. taiwanensis* and *P. putida* strains have applications in genetic engineering and biotechnology, for example as a basis for the synthesis of secondary metabolites (Schwanemann 2023, Gross 2006, Loeschcke 2015, Bretschneider 2021).

As part of the research, two bacterial strains from anthropogenically degraded environments were isolated, which in the course of the presented research were identified as *Pseudomonas alloputida* and *Pseudomonas taiwanensis*. Due to the documented ability of both microorganisms of decomposing complex substances, the research determined their activity in the production of hydrolytic enzymes involved in the degradation of polymer materials. Also, the effect of degrading bacteria on the PLA film was examined. Thus, the hypothesis that these strains have a positive effect on improving and accelerating the biodegradation of plastics under mesophilic conditions was verified.

2. Materials and methods

2.1 Polymer material

The PLA foil was extruded from IngeoTM 2003D granules (NatureWorks LLC, USA) using a Plasti-Corder PLV 151 single-screw extruder (Brabender, Germany). Before processing, the granules were dried for 8 hours at 80 °C. A film with a thickness of 0.09 mm ± 0.02 mm was obtained from which samples of 100 mm × 10 mm were prepared.

2.2 Bacteria isolation

The studied microorganisms were isolated from anthropogenically degraded environments, i.e. from landfill soil and activated sludge. Subsequently, the bacterial strains were identified, and a patent deposit was filed in the national collection.

2.2.1 Isolation from soil

Environmental soil samples were prepared from a landfill (Niedźwiedź, Poland). The sample was homogenized, and then diluted, in which the PLA film was placed. The samples were incubated at 26 °C ± 2 °C with continuous shaking for 28 days. Bacterial cultures were obtained by swabs from the PLA foil with addition of fungal antibiotic nystatin. From the obtained cultures, single bacterial colonies were collected, which, based on morphology assessment, were the most abundant. According to the hypothesis, it could indicate that the polymer material was treated as an adhesion surface, and subsequently as a carbon source. The collected colonies were inoculated on agar medium until an isolate with a homogeneous morphological structure was obtained. The morphologically homogeneous sample was designated KJ-LIN-3-1 and directed for genetic identification by Sanger DNA sequencing based on 16S rRNA. The sequence of the KJ-LIN-3-1 sample showed 100% similarity to *Pseudomonas taiwanensis* (GenBank: KM349421.1, strain P5). Strain was deposited in the Institute of Agricultural and Food Biotechnology State Research Institute (IAFB) Collection of Industrial Microorganisms under name of KKP 2098p.

2.2.2 Isolation from activated sludge

Environmental samples from activated sludge were prepared. The sludge was cultured by mechanically passing it through sieves with a mesh size of 0.2 mm. The wastewater was aerated for 2-3 days in the reactor. The sample was homogenized for 40 seconds at a speed of 24,000 rpm. Then the procedure was carried out as in section 2.2.1 until an isolate with a homogeneous structure was obtained, which was designated as KJ-OS-5-3. The sequence of the KJ-OS-5-3 sample showed the highest similarity to *Pseudomonas alloputida* (GenBank: CP128550.1, strain NMI5594_09). The strain was deposited in IAFB (Poland) under number KKP 2097p.

2.2.3 Assessment of hydrolytic activity of identified bacteria

The ability of the identified bacteria to produce hydrolytic enzymes: proteases, amylases, cellulases and pectinases was investigated based on bacterial cultures in a medium with a given substrate as the only carbon source.

2.2.3.1 Proteolytic activity

Bacteria were cultured on a medium with the addition of casein as the only source of carbon according to Burbianka and Pliszka (1983). Plates were incubated for 4 days at 26 °C ± 1 °C. Halo (clear) zones were observed on the medium around the grown colonies, which indicated hydrolysis of casein.

2.2.3.2 Cellulolytic activity

The ability to produce cellulases was tested on a nutrient solution according to Wood (1980) with the addition of carboxymethyl cellulose (CMC). The inoculated plates were incubated for 4 days at 26 °C ± 1 °C. The hydrolysis capacity of CMC was determined by pouring 0.1% Congo red solution over the culture plates. After 15 minutes, the plates were washed with tap water and poured with 1 M NaCl solution for 15 minutes. Clear zones around the colony indicated that hydrolysis had taken place (non-hydrolysed CMC remained red).

2.2.3.3 Amylolytic activity

The ability to hydrolyze starch was investigated on a medium with the addition of starch according to Gibson and Gordon (1974). The inoculated plates were incubated for 4 days at 26 °C ± 1 °C. The ability to decompose starch was checked by pouring Lugol's solution over the culture plates and observing the halo zones around the colony.

2.2.3.4 Pectinolytic activity

Pectinolytic activity was investigated by bacterial culture on a medium with the addition of citrus pectin according to Strzelczyk and Szpotański (1989). The hydrolysis capacity was determined by pouring 1% Cetrimide (cetyltrimethylammonium bromide, BDH) solution over the culture plates, which precipitated the non-degraded pectin. Halo zones around the bacterial colonies indicated that hydrolysis had occurred.

2.3 Assessment of PLA foil biodegradation by *P. taiwanensis* and *P. alloputida*

For each of the isolated cultures of the identified microorganisms (KJ-LIN-3-1 and KJ-OS-5-3), bacterial suspensions of 1.2 – 1.5 × 10^4 cfu/ml were made, which were added to a liquid medium without a carbon source prepared in accordance with ISO 846 (2019). Sterile fragments of PLA foil, which were the only source of carbon, were added to the solution. Samples were incubated for 28 days at 26 °C ± 2 °C with shaking. An uninoculated liquid medium with the addition of PLA was used as a control sample. After incubation, the PLA samples were removed, dried and structural and mechanical analyses of the material were performed. Values of pH, redox potential and Total Bacteria Count (cfu/ml, TBC) in the medium were examined.

2.3.1 Infrared Fourier analysis

FTIR spectra were recorded in the range of 400–4000 cm^{-1} using a Cary 630 FTIR spectrometer (Agilent Technologies, USA) combined with ATR method (Attenuated Total Reflection),. Based on the ratio of absorbance at 1746 cm^{-1} wavelength to absorbance at 1450 cm^{-1} wavelength, carbonyl index was determined. Lower ratio of carbonyl index values indicates the degradation of the material due to decreasing number of carbonyl bonds. Results were compared to control samples.

2.3.2 Microscopic analysis

PLA samples were analysed using scanning electron microscopy (SEM, Hitachi, Japan). The surface of the foil was observed at a magnification of ×1000 at a voltage of 5-10 kV. Energy dispersive X-ray analysis was performed using SEM-EDX (Thermo Scientific Ultra Dry, USA) and O/C ratios of the surface samples were calculated. Higher values of O/C ratio indicate deterioration of sample structure due to loss of mass carbon from polymeric material according to Puszczykowska et al. (2022).

2.3.3 Mechanical properties

Mechanical properties: force at break - FB (N) and elongation at break - ε (%) of degraded samples were determined using TIRATest 27025 equipment (TIRA GmbH, Germany) according to international standard (ISO 527-1, ISO 527-3).

3. Results

3.1 Hydrolytic activities of *P. taiwanensis* and *P. alloputida*

Hydrolytic activities of the studied bacterial strains are summarized in Table 1. Results indicate high hydrolytic activity against all substrates in the case of *Pseudomonas taiwanensis*. *Pseudomonas alloputida* hydrolyzes three of the four substrates studied.

Table 1: Summary of produced enzymes by identified bacteria.

Produced enzymes	*P. taiwanensis*	*P. alloputida*
Protease	+	+
Cellulase	+	+
Amylase	+	+
Pectinase	+	−

Abbreviations:
+ - observed hydrolysis zone around colonies, bacteria producing hydrolase,
− - not producing hydrolase.

3.2 Assessment of PLA foil biodegradation by *P. taiwanensis* and *P. alloputida*

3.2.1 Characteristics of liquid culture

The culture medium characteristics was assessed to control and provide the conditions of optimal bacteria growth. In both cases, a high increase in the number of bacterial cells per milliliter was determined. The pH value of the liquid culture decreased slightly, while the redox potential increased.

Table 2: Initial and final values of pH, redox potential and TBC of the incubation liquid medium

	P. taiwanensis culture	*P. alloputida* culture
Initial pH value	6.552	6.511
Final pH value	6.463	6.369
Initial redox potential [mV]	190.6	182.8
Final redox potential [mV]	203.2	211.3
Initial TBC [cfu/ml]	1.2×10^4	1.5×10^4
Final TBC [cfu/ml]	2.7×10^8	2.1×10^8

3.2.2 Carbonyl index and O/C ratio

The carbonyl index and O/C ratio was determined for each foil sample. The arithmetic mean and standard deviation were calculated on Figure 1. The FTIR-ATR analysis showed a decrease in the carbonyl index of the PLA samples as a result of the incubation with both bacterial strains compared to the control sample, while a lower value was noted for *Pseudomonas alloputida*. The O/C ratio of the tested samples increased compared to the control sample and a higher value was noted in the case of *Pseudomonas taiwanensis*.

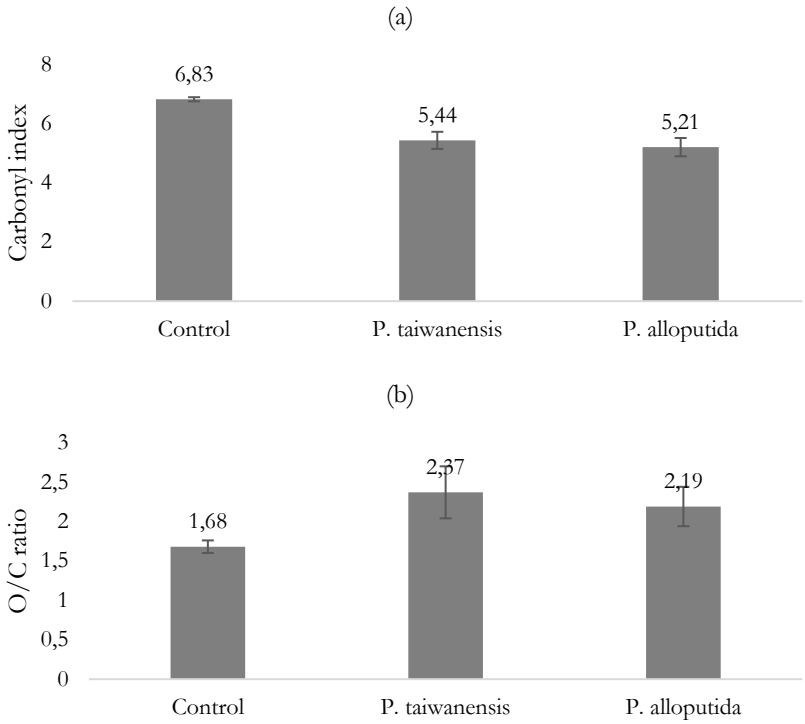
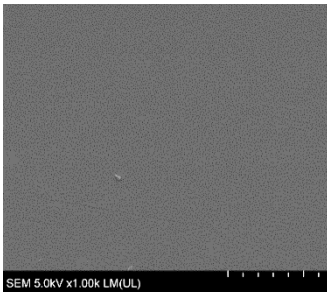
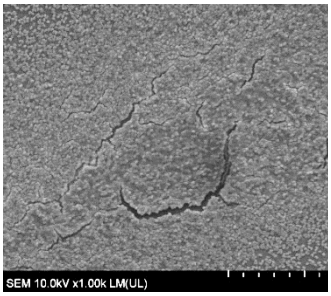
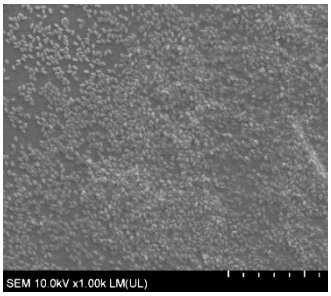


Figure 1: Arithmetic mean with standard deviation of calculated (a) carbonyl index and (b) O/C ratio of biodegraded PLA samples.

3.2.3 Microscopic observations

Microscopic observations of the surface of the control sample, the incubated sample from *Pseudomonas taiwanensis* and *Pseudomonas alloputida* was performed. Strong bacterial growth and biofilm formation were observed on the surface of the samples (Table 3).

Table 3: Representative images of surface of post-incubated PLA samples.

Control	PLA incubated with *P. taiwanensis*	PLA incubated with *P. alloputida*
		

3.2.4 Mechanical properties

Deterioration of the material of the test samples compared to the control sample was demonstrated. The values of force at break and elongation at break decreased especially after incubation of samples in the presence of *P. taiwanensis*. For control samples, the force at break value was 87.14 N, after incubation with *P. alloputida* 70.7 N, and after incubation with *P. taiwanensis* 62.58 N (Figure 2). The elongation at break value for the control sample was 5.16% and after incubation with *P. alloputida* and *P. taiwanensis*, respectively 3.19% and 2.88%.

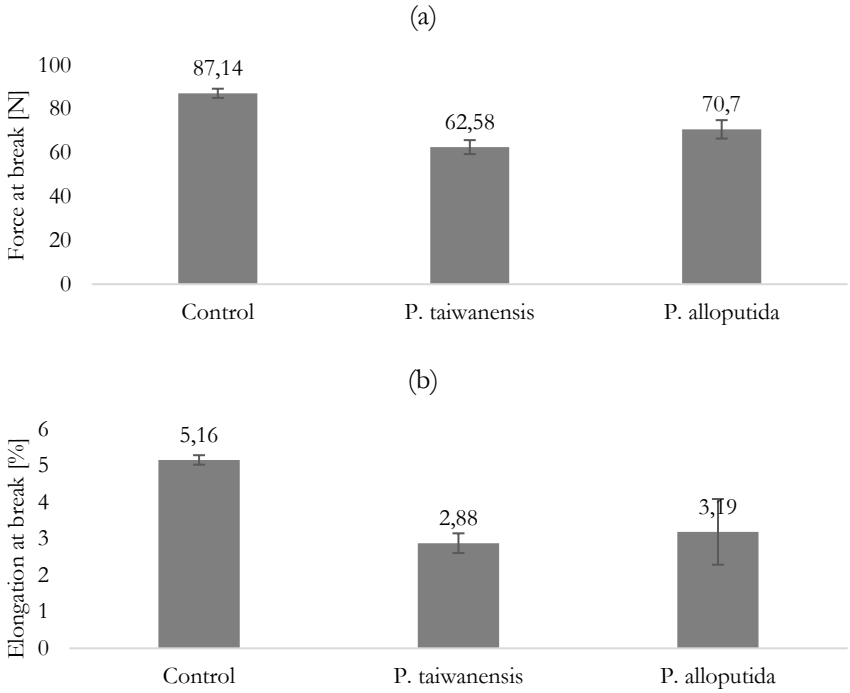


Figure 2: Values with standard deviation of (a) force at break (FB) and (b) elongation at break (ε) of degraded PLA samples.

4. Discussion

In order to select microorganisms effective in accelerating the process of PLA biodegradation, two strains of bacteria belonging to *Pseudomonas* were isolated and genetically identified: *P. taiwanensis* corresponding to the sequence of the previously described strain designated P5 and *P. alloputida* designated by other researchers as NMI5594_09. No documented reports of the results of similar studies on the use of isolated bacterial strains in the degradation of polymer materials have been found.

Bacteria of the genus *Pseudomonas* are a unique group due to the classification of many of them as plant-growth promoting rhizobacteria (PGPR) (Singh 2022). These bacteria colonize plant roots fixing nitrogen and producing siderophores. A number of reports describe strains belonging to *Pseudomonas* due to wide enzymatic activity. For

instance, producing (ACC)-deaminase, facilitates acclimatization to drought (Arshad 2008). PGPR-*Pseudomonas* produce auxins, cytokinins, gibberellins and abscisic acid, thus regulating the processes of acclimatization and adaptation to environmental stress (Singh 2022, Georgieva 2018, Kumar 2018). For these reasons, it can be concluded that the identified microorganisms will not adversely affect the environment of the biodegradation process (e.g. composting) and thus the quality and safety of the processed organic matter.

Conducted studies indicate high hydrolytic activity of *P. taiwanensis* and *P. alloputida* strains in relation to substrates: casein, cellulose and starch. Citrus pectin is hydrolyzed only by *P. taiwanensis*. High hydrolytic capacity is a sign of correctly performed bacterial selection. From all of the properties studied, proteolytic activity is the most important, as the literature provides many examples where proteases are used in biodegradation of polymer materials (Oda 2000, Tokiwa 2006). As early as 1994, it was shown that polylactide can be degraded by proteinase K (Reeve 1994). However, Gan et al. showed a strong degradation effect of the lipolytic enzyme against another biodegradable polymer, poly(ε-caprolactone). The selected strains demonstrated very high enzymatic activity and thus the potential to accelerate degradation in laboratory conditions. The obtained results have a chance of being successfully used for environmental research. However, the challenge remains to adapt the appropriate method of bioaugmentation to soil or compost. Aspects such as the CFU values of bacteria, form, how to ensure survival among the numerous autochthonic microflora, the amount of polymer pollution that may be present in the environment for the process to be efficient and others should be considered and need further examination.

In liquid culture of both strains in the presence of PLA, an increase of approx. 10^4 cfu/ml was observed, which indicates a tendency for the tested bacteria to multiply in the presence of PLA. Survival and multiplication in an environment where polymer is the only source of carbon confirms the potential of selected bacterial strains, and their appropriate abundance increases their effectiveness. The potential of *Pseudomonas* strains to colonize extreme environments exposed to stress factors such as salinity, drought, and the presence of aromatic hydrocarbons has been confirmed, among others, by Singh et al. (2022).

The pH value of the liquid culture of both bacterial strains in the presence of PLA decreased slightly, which may indicate acidification of the medium through hydrolytic degradation of the foil and thus the release of lactic acid residues. Similar results were obtained by Elsawy et al. (2017) and Gorrasi et al. (2018), who observed that a decrease in pH may also indicate bacterial metabolic activity and the associated release of larger amounts of CO_2. Undoubtedly, changes in pH affect the abiotic and biotic factors of the soil. The activity of most bacterial strains is greatest at pH 6-8. As literature indicates, optimal pH for biodegradation of aromatic compounds by *Pseudomonas* sp. is at pH value 8 (Khan 2009). This fact may suggest that in natural conditions, biodegradation of PLA in soil environment, where pH ranges usually from 6 to 9, would occur in very high rate. Enzymatic activity also depends on the pH of the soil substrate, while hydrolytic enzymes have an optimum in ranges of similar values. For example, proteases from pineapple extract have optimal specific activity at pH 6 and 7 (Silvestre 2012). As pH level changes, the availability of macro- and micronutrients also changes (Neina 2019). Therefore, a change in pH affects a number of different factors on which the further course of the

degradation process may depend. It is assumed, however, that in the event of further decomposition of PLA in this experiment, further acidification of the soil substrate would occur and then the development of acidophilic bacteria. For this type of microorganisms, lactic acid, which is the building block of PLA, could be used for metabolic processes. Moreover, the process would take place in conditions of limited competition. An increase in the redox potential values indicates oxidative processes taking place in the culture and is also an indicator of the activity of microorganisms.

The value of the carbonyl index of the incubated foil with *P. taiwanensis* decreased by 20.4% compared to the control, and the value of the incubated PLA with *P. alloputida* by 23.7%. The results indicate a significant loss of carbonyl bonds in the polymer material and its deterioration related to biological activity (Bajer 2007, Sudhakar 2008, Janczak 2018, Szczyrba 2022). Observations of the surface of the samples after incubation confirm the ability of the tested strains to form a biofilm. Researchers analysing the formation of biofilm on the surface of polyethylene have shown a strong relationship between colonisation and the degree of biodegradation of the material. This is due to the increased ability of microorganisms to utilise the carbonyl residues that form on the surface of the material (Hadar 2004). Biofilm-forming bacteria form a cluster surrounded by extracellular polymeric substances (EPS) that protects the bacterial colony and thus contribute to the optimal growth of bacterial cells. Then, bacteria are able to attach to a favorable surface (in this case polylactide) even in unfavorable conditions. The biofilm matrix, consisting mostly of exopolysaccharides, proteins, nucleic acids and humic acid substances, protects the colony against the negative influence of the environment (Mahto 2022). Bacterial adhesion enables enzymatic cleavage of the polymer chain, causing the release of oligomers and, ultimately, monomers, which are utilized by individual cells. Therefore, the ability of the tested bacteria to form a biofilm has a positive effect on the biodegradation of PLA.

The research focused on mesophilic conditions, which was an additional challenge. There are many works on the effective composting of polymer materials, including PLA, emphasizing the role of high temperature. However, we are only able to achieve thermodegradation under industrial composting conditions. In home composters, the temperature is usually maintained at around 25 °C, hence similar experimental conditions were used. Average temperatures in such composters can range from 12.5 to 27.4 ºC (Vázquez 2017). It is known that in such conditions bacteria of the *Pseudomonas* sp. are able to survive and function metabolically (Cullen 1971, Membré 1994). The aim of further research is to develop a bioproduct that could also be used in home composting conditions. In order to conduct a detailed study of the selected strains, their pH, temperature tolerance range and required nutrients will be determined.

The values of the O/C mass ratios in the surface structure of the biofilm-cleaned, degraded foils increased by 41% in the case of incubation with *P. taiwanensis* and by approx. 30% with *P. alloputida* compared to the control. The results indicate a decrease in the amount of carbon molecules from the polymer material, which may indicate the assimilation of carbon by microorganisms as a result of enzymatic reactions. Similar conclusions were reached by Zumstein et al. (2018), who studied the microbial colonization of poly(butylene adipate-co-terephthalate) (PBAT) in soil. By bacterial enzymatic depolymerization, carbon from the polymer structure was assimilated and converted into CO_2 and biomass.

Studies of the mechanical properties of the material allowed to observe a decrease in the tested parameters, and thus a decrease in the quality of the material as a result of biodegradation. Analysis of polymeric materials for force at break (FB) and elongation at break values is a common procedure to assess the influence of various factors on its strength (Shibata 2004, Lee 2014).

In the course of subsequent studies including *in situ* tests, it may turn out that the hydrolytic activity of the strains is insufficient to make the process economically profitable in terms of the amount of degraded waste. The solution may be biotechnology and genetic engineering, the possibilities of which can improve the metabolic activity of the tested strains, and thus improve the biodegradation of polymer materials. Scientists report on enhancing the metabolic activity of cellulase-producing *Pseudomonas* strains (Bakare 2005) and increasing the production of proteases (Dutta 2006). Modification in terms of additional features allowing adaptation to abiotic environmental factors, such as high or low temperature, salinity, presence of heavy metals, would optimize bacterial multiplication. Literature indicates research on *Pseudomonas* sp. with high tolerance to high temperature, drought (Chaudhry 2015) or adaptation to saline stress (Costa-Gutierrez, 2020).

In addition to biotechnological challenges, the implications of the research require focusing on the ecological and economic aspects. The proposed solution of introducing active bacteria to compost cannot have a toxic effect on the properties of the produced matrix during composting which is by design introduced again to the environment. To reach that, the lack of phytotoxicity of the obtained compost should be demonstrated. If a large amount of PLA waste is degraded, as stated above, acidification of the soil substrate may occur. In this case, the compost can probably be used to grow a limited number of plant species. However, there are substrates on the market dedicated to plants growing in acidic soil. It is believed that among those considered biodegradable, PLA is the most difficult to degrade. In order to increase the potential of the product, it is necessary to analyze the effect of given *Pseudomonas* strains on other polymer wastes present in real conditions. To produce the mixture of bacteria on a large scale, bioreactors can be used, which are considered economically viable and are used for various bacterial products, for example biogas or bacterial polymers such as PHA (Sabra 2010). We believe that these mentioned solutions contribute to sustainable polymer waste reduction in the soil and compost environment.

5. Conclusions

Obtained results indicate a significant potential of anthropogenically degraded environments as places where microorganisms with high enzymatic activity are present. Strains belonging to *Pseudomonas*: *P. taiwanensis* and *P. alloputida* have hydrolytic abilities and form a biofilm on the surface of the polymer material, which allows them to effectively decompose polymeric materials such as PLA. These bacteria can be implemented into the soil or compost as a factor accelerating the cleaning of the environment from polymer plastic waste, in particular those made of PLA. The next stage of the research will be the preparation for application tests, including the selection of the most appropriate number of microorganisms and polymer waste to ensure the effectiveness of the process under

composting conditions, as well as evaluating the effectiveness of *P. taiwanensis* and *P. alloputida* strains against other biodegradable polymer materials found in the organic waste. The limitation of given solution is presence of conventional, non-biodegradable polymer waste made of materials such as polyethylene, polypropylene or polystyrene, for which mechanical separation from organic waste is still the most effective method. Hence, an extremely important aspect is to constantly increase social awareness about biodegradable and non-biodegradable materials.

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