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SCREENING OF CULTIVATION MEDIA FOR LDPE BIODEGRADATION BY *Pseudomonas fluorescens*

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Abstract

The research aimed to select the optimal mineral salt medium (MSM) for low-density polyethylene (LDPE) biodegradation by the strain Pseudomonas fluorescens CNM-PFB-01. Four culture media were selected, which differed in salt content, N: P and C: N ratio. After 40 days of submerged cultivation, the following parameters were determined: catalase activity, pH of culture media, biomass accumulation, rate of degradation and the tensile tests of LDPE. It was observed that the strain is catalase-negative both in the control without LDPE and in the presence of LDPE. A weak positive reaction was established only on MSM 4 supplemented with LDPE. In the presence of polyethylene, the pH of the media increased, especially on the MSM 4 - by 0.6 units. The addition of polyethylene to the growth media stimulated the bacterial biomass accumulation by 2-3.6 times. The degradation rate of polyethylene films ranged from 0.37% to 0.86% depending on the culture medium. The tensile test showed increased elasticity of the plastic in the variants treated with bacterial strain. In conclusion, in order to stimulate the biodegradation of LDPE by the strain P. fluorescens CNM-PFB-01, the medium MSM 4 (N: P ratio 4.30: 1 and C: N ratio 0.29: 1) was selected.

Key words*: mineral salt media, catalase activity, LDPE biodegradation, Pseudomonas fluorescens.*

INTRODUCTION

Bacteria of the genus *Pseudomonas*, which are widely spread both in soil and in aquatic environments, are recognized as a valuable source for modern biotechnology, due to their varied metabolic capacities (Wilkes & Aristilde, 2017). Over the decades, where the world is looking for ecological remedies to destroy xenobiotic substances, the metabolic properties of pseudomonads have been used in bioremediation of a wide spectrum of pollutants of different nature, such as organochlorine pesticides, petroleum hydrocarbons, phenolic compounds, heavy metals, plastic polymers, etc. (Wasi et al., 2013).

In terms of plastic bioremediation, besides fungi of the genus *Aspergillus* and *Penicillium*, bacteria of the genus *Pseudomonas* are among the most cited in literature as destructors of different plastic polymers (Kyaw et al., 2012; Wilkes & Aristilde, 2017). Thus, among the bacteria that degrade polyvinyl alcohol, most of them are pseudomonads (Shimao, 2001). There are data that pseudomonads contribute to the degradation of polyethylene succinate (Tribedi & Sil, 2013a), polystyrene (Devi et al., 2016) polypropylene (Arkatkar et al., 2010). Complete degradation of polyethylene glycol was obtained using *Pseudomonas stutzeri JA1001* by Obradors & Aguilar (1991). Also, several strains of pseudomonads including *P. fluorescens*, *P. aeruginosa*, *P. cepacia*, *P. protegens* and *P. chlororaphis* have been found to degrade polyurethane (Cregut et al., 2013).

One of the most used plastic materials in the world economy - polyethylene (PE), is characterized by stability and durability due to hydrophobic carbon backbone, which makes it recalcitrant to the action of external factors, including biological ones. However, strains of pseudomonads capable of degrading PE, from 5% to 50%, have been detected, depending on the structure of the PE, its pretreatment and the type of the *Pseudomonas* strains (Rajandas et al., 2012; Tribedi & Sil, 2013b).

The main strategy in bioremediation of LDPE is based on the use of plastic by the microorganism as sole carbon source. For this, microorganisms synthesize enzymes, which trigger the oxidation processes of carbon bonds. The duration and degree of degradation of PE

depends both on the structure of the polymer surface and on the environmental conditions (pH, temperature, nutrients, minerals, oxygen, humidity etc., as well as on the physiological properties of each individual microorganism) (Dwicania et al., 2019; Iram et al., 2019; Tamnou et al., 2021).

For these reasons, the selection of nutrient media and cultivation conditions, which would ensure the maximum triggering of the physiological processes of the microorganism, aimed to use plastic as a source of carbon, is an important step in bioremediation technologies. Thus, the aim of our research was to select the optimal mineral medium for LDPE biodegradation by the strain *Pseudomonas fluorescens* CNM-PFB-01.

MATERIALS AND METHODS

The object of study was bacterial strain *P. fluorescens* CNM-PFB-01 deposited in the National Collection of Non-Pathogenic Microorganisms of the Republic of Moldova. LDPE sheets used in this work were produced by Kraus Folie Sp.J. Film thickness was 35 µm. Four mineral salt media (MSM) were selected which differed in salt content (Table 1), and N: P and C: N ratio (Table 2).

Table 1. The composition of the mineral media, g/L

Mineral salts, g/L	**MSM 1** (Jamil, 2017)	**MSM 2** (Nakei, 2019)	**MSM 3** (Skariya-chan, 2015)	**MSM 4** (Zajic, 1972)
KH_2PO_4	2.0	1.0	2.0	
K_2HPO_4	7.0	1.0	7.0	1.8
$MgSO_4 \cdot 7H_2O$	0.1	0.2	0.1	0.2
$ZnSO_4 \cdot 7H_2O$	0.001			
$FeSO_4 \cdot 7H_2O$	0.01			0.01
$MnSO_4 \cdot 6H_2O$	0.002			
NH_4NO_3	1.0	1.0		
$CuSO_4 \cdot 7H_2O$	0.0001			
$CaCl_2$		0.02		
$FeCl_3$		0.05		
$(NH_4)_2SO_4$			1.0	
$NaNO_3$				2.0
NH_4Cl				4.0
NaCl				0.1
$Na_3C_6H_5O_7$			0.5	

Table 2. Nitrogen, phosphorus, and carbon content and ratio in the tested mineral media

Mineral media	Nitrogen, g/L	Phos-phorus, g/L	Carbon, g/L	Ratio N: P	Ratio C: N
MSM 1	0.35	1.70	0.40	0.21	1.14
MSM 2	0.35	0.41	0.40	0.86	1.14
MSM 3	0.21	1.70	0.54	0.12	2.55
MSM 4	1.40	0.32	0.40	4.30	0.29

The composition of the selected media was formulated so that the suspended polymer, LDPE, was the sole carbon source for the microorganism. As a growth inducer, 0.1% glucose was added to the medium. The pH of the media was adjusted to 6.5, according to the physiological needs of the bacterium.

Each medium had 2 experimental variants - with LDPE films and without LDPE films (control variant). After 40 days of submerged cultivation at 28°C, on a shaker (180-200 rpm), the following parameters were determined: catalase activity and pH of culture media, quantity of accumulated biomass, the rate of LDPE degradation, optical microscopy of films.

Also, the tensile testing of polyethylene (ISO 20753:2008) was performed using the tensile testing machine CQ-508B (COMETECH Testing Machines Co., LTD). Such parameters as elongation at break (%) and tensile strength at break (N/mm^2) were determined.

The catalase test of culture media was performed by the slide method using 3% H_2O_2.The reaction was interpreted according to the intensity of the formation of oxygen bubbles.

Before adding to culture media, LDPE films were cut into longitudinal and transverse strips, weighed and sterilized by washing with 70% ethyl alcohol for 15 min, and treated with UV-rays for 1 hour twice. After 40 days of cultivation, the LDPE films were recovered, washed with sterile distilled water, air-dried and examined under an optical microscope.

The degradation of LDPE films was determined gravimetrically, by weighing the films before and after incubation. The bacterial cell mass adhering to the polyethylene surface was washed by a 2% aqueous sodium dodecyl sulfate solution for 3 hours and finally with distilled water. The washed LDPE films were

air-dried and weighed. Percentage degradation of polyethylene films was determined by the formula:

Weight loss (in %) = [(Initial Weight − Final Weight) / Initial Weight] × 100.

RESULTS AND DISCUSSIONS

The first stage in the biodegradation of plastic consists in the attachment of microorganisms on the surface of the polymer, followed by the increase of biomass and the synthesis of specific enzymes (Montazer et al., 2020; Wilkes & Aristilde, 2017; Alshehrei, 2017). At the end of the cultivation period of the *P. fluorescens* CNM-PFB-01 strain on different mineral media with and without addition of LDPE films, the catalase activity and the pH of the cultural media were determined (Table 3).

Catalase is a commonly assayed enzyme that degrades hydrogen peroxide into water and oxygen. The presence of the enzyme in the test bacterial isolate or culture medium can be determine by using hydrogen peroxide, which is broken down to bubble-producing O_2 by catalase-positive bacteria (Iwase et al., 2013). The measurement of bacterial catalase activity has been suggested as a method to quantify catalase-positive bacterial content (Serra et al., 2008). There are reports discussed determination of aerobic microbial concentrations based on the correlation between catalase activity and aerobic microbial loads (Ye & Wu, 2011). In our work we used catalase test for rapid assessment of viability of microbial culture. *Pseudomonas* species also typically give a positive result to the catalase test. It was established that the strain is catalase-negative both in the control variants and in the presence of LDPE. The exception is the variant MSM 4 with LDPE, where a weak positive reaction was observed.

The researchers noted that the bacteria *Pseudomonas* grows best in the pH range of 6.3-7.2 (Stoimenova et al., 2009; Gonçalves et al., 2017; Bushell et al., 2019). Over time, the pH of the culture medium changes towards its acidification due to the accumulation of organic acids, as well as metabolic products synthesized by these bacteria during cultivation (Zhou et al., 2017). The evolution of the pH of the culture medium speaks of the metabolic activity of the microbial strain in the medium supplemented with LDPE, and is important for enzymatic activity, since it has been demonstrated that more alkaline pH favors enzyme activity (Dwicania et al., 2019; Iram et al., 2019; Tamnou et al., 2021). Considering physiological requirements for growth of pseudomonads, the pH of media used was initially adjusted to 6.5. After 40 days of cultivation, the pH remained practically unchanged on the MSM 3 (values - 6.3-6.4), but decreased to more acidic values of 5.4-5.6 on the MSM 2, and 5.6 on the MSM 4 without LDPE. Unlike the other media, there was a large difference between the pH values on the MSM 4 with and without LDPE - by 0.6 units (Table 3).

Table 3. Catalase activity and pH of the cultural media after cultivation of *P. fluorescens* CNM-PFB-01 on different MSM with and without plastic

Nutrient medium	Catalase activity		pH of culture media	
	Without LDPE	With LDPE	Without LDPE	With LDPE
MSM 1	-	-	6.1	6.2
MSM 2	-	-	5.4	5.6
MSM 3	-	-	6.3	6.4
MSM 4	-	+	5.6	6.2

N.B.: - No reaction, + Weak reaction

It is well known that the ratio of carbon to nitrogen is an important indicator for the growth and biosynthesis of bacteria (Ginésy et al., 2017). In this work, the source of carbon for the growth of pseudomonads was the polymer LDPE, while glucose at a concentration of 0.1% and sodium citrate (as a component of the MSM 3 medium) served as a growth inducer. Thus, the amount of available carbon was sufficient to initiate bacterial growth, but not enough to accumulate a large amount of biomass, as in the case of growth on media

with a high C: N ratio, as seen in examples from the literature (Hartmann et al., 2004; Onwosi & Odibo, 2012; Veliev et al., 2013). It was observed that the presence of LDPE in the culture media considerably influenced the growth activity of the strain - the amount of accumulated biomass doubled or even tripled (Figure 1).

Thus, on MSM 1 the amount of biomass increased by 2 times, on MSM 2 - by 2.9 times, and on MSM 4 - by 3.6 times. The exception occurred on MSM 3 supplemented with LDPE, where the strain accumulated less biomass than in the control by 13.8%. This medium characterized by C: N ratio 2.55 and N: P ratio 0.12 (limitation on phosphorus).

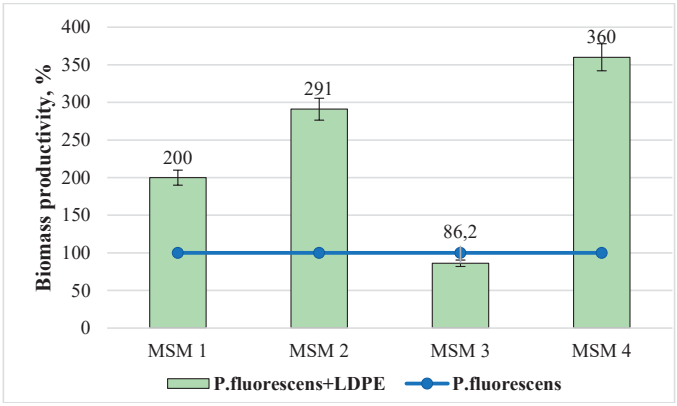


Figure 1. Accumulation of *P. fluorescens* CNM-PFB-01 biomass on different mineral media, with and without LDPE

To assess the degradation of plastic we used such indices as weight loss of the substrate and changes in mechanical properties (tensile strength, percentage of elongation).

The degradation of plastic by bacteria of the genus *Pseudomonas* is intensely studied. In the literature, data showing that the percentage of mass loss of polyethylene varies widely is presented. Thus, *Pseudomonas* sp. AKS2 strain degraded up to 5% LDPE (Tribedi & Sil, 2013b), and *P. aeruginosa*_UMT - 4.8% (Bakht et al., 2020). A series of actively degrading LDPE pseudomonads (with 20% *P. aeruginosa* (PAO1), 11% *P. aeruginosa* (ATCC), 9% *P. putida*, and 11.3% *P. syringae*) were detected by Kyaw et al. (2012). The conditions, nutrition media and duration of cultivation in the listed cases were different from those presented in this paper.

Weighing the LDPE strips after 40 days of cultivation showed that in the presence of *P. fluorescens* CNM-PFB-01 the strips lost mass and the degradation processes of plastic had begun. The percentage of degradation was different, depending on the culture medium, from 0.37% to 0.86% (Figure 2).

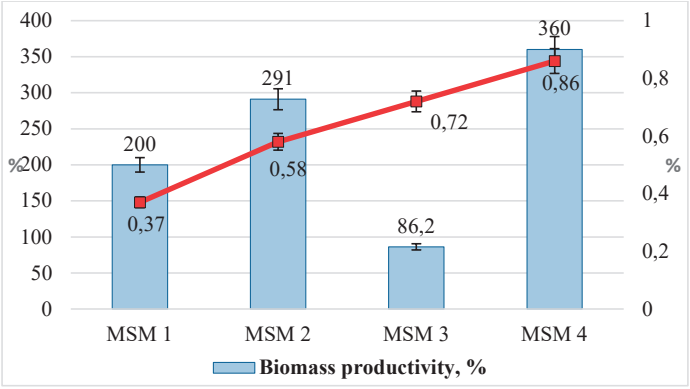


Figure 2. LDPE degradation and the amount of *P. fluorescens* CNM-PFB-01 biomass on different mineral media

It was observed in the case of MSM 1, MSM 2 and MSM 4 that the percentage of degradation is directly correlated with the amount of biomass: the more biomass the strain accumulated, the higher the percentage of degradation was. On MSM 3, although the strain growth was diminished due to limitation on phosphorus, LDPE degradation was found to be more active than on MSM 1 and 2. The most active the LDPE films were degraded on medium MSM 4, with the lowest C: N ratio and the highest N: P ratio. The effect of colonizing the polyethylene surface with microorganisms can also be evaluated by determining the mechanical properties of the film, namely by determining the tensile strength and elongation at break. The higher the tensile strength of the polymer, the better its stability. According to the literature data microorganisms affect the mechanical properties of LDPE leading to a change in tensile strength and elongation at break (Sudhakar et al., 2008; Nowak et al., 2011; Kyaw et al., 2012; Ghatge et al., 2020). In our case, the tensile tests on polyethylene showed that the elasticity of the films increased in the samples processed with microorganisms (Figure 3).

Thus, compared to the untreated LDPE films with pseudomonads, the elongation at break of the transverse stripes changed considerably on all tested media by 21-48% (maximum on MSM 1 - by 48%), and the tensile strength by 4.5-21%. The most visible changes in the elasticity of the plastic were determined on the MSM 1 medium.

The elasticity for the longitudinal stripes has not changed so much. On MSM 1 and 3 media, the values of the elongation at break and tensile strength varied within the control limits. Visible changes of the longitudinal film were established when cultivated on MSM 2, the value of the elongation at break increased by 32% and of the tensile strength by 24%.

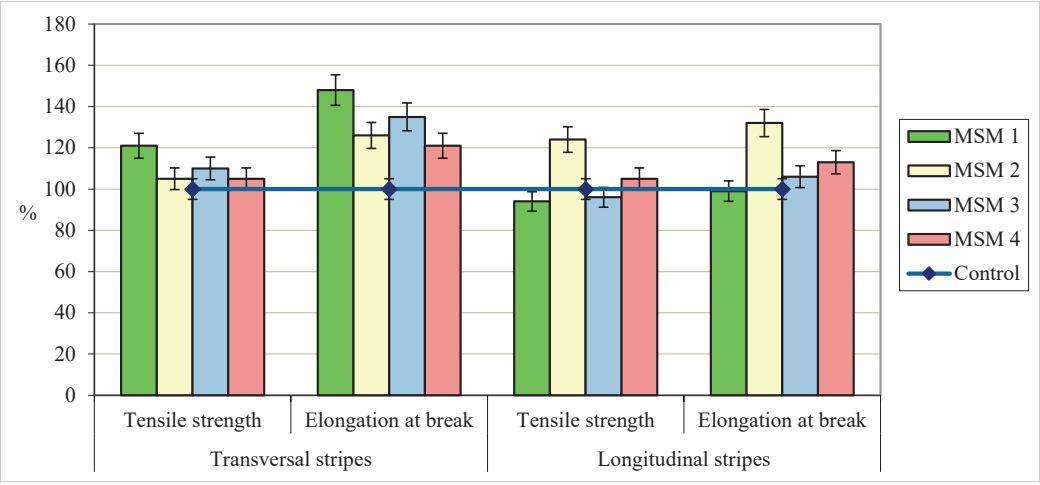
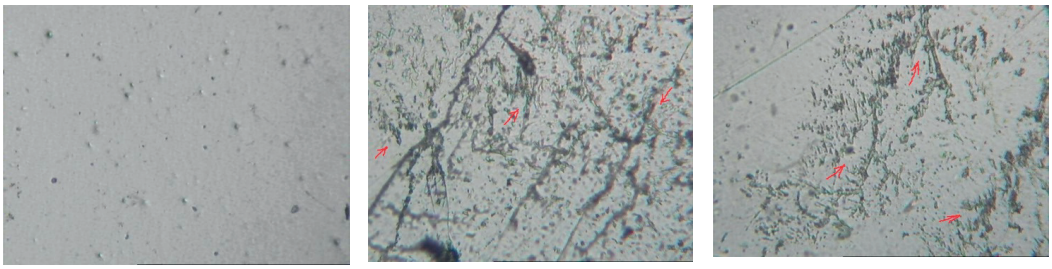


Figure 3. The tensile strength and elongation at break of LDPE films treated with *P. fluorescens* CNM-PFB-01, depending on the culture medium

The process of biodegradation of plastic begins with the colonization of its surface by microorganisms (Kyaw et al., 2012; Ghatge et al., 2020). The microscopy of the LDPE films revealed that the bacterial cells became immobilized on the surface of the films, and the contact damaged the polyethylene surface (Figure 4).



Untreated polyethylene (Control) Traces of damage on the surface of polyethylene

Figure 4. Photo of LDPE films after treatment with *P. fluorescens* CNM-PFB-01

CONCLUSIONS

Of the 4 tested mineral media, it was observed that the most optimal conditions for the active growth of the strain *P. fluorescens* CNM-PFB-01 were created at cultivation on MSM 4, where the C: N ratio was 0.29:1 and N: P ratio was 4.30: 1. Due to the alkalinization of the medium, the synthesis of the catalase ferment was triggered, which in turn favored the initiation of the degradation process of LDPE film. MSM 4 medium was selected for further research.

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